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Sprouty4 regulates the balance between pluripotency and trophectoderm differentiation in mouse embryonic stem cells

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Summary

A hallmark feature of embryonic stem (ES) cells is the ability to self-renew indefinitely while maintaining pluripotency. However, the molecular and cellular mechanisms underlying ES cell fate are poorly understood. To identify signaling pathway molecules that maintain the uncommitted state of ES cells, a microarray analysis was performed comparing undifferentiated ES cells, mature embryoid bodies, spontaneously differentiated and retinoic acid-induced differentiated ES cells.

Among several well-validated pluripotency markers, *Sprouty4* was identified as one of the most highly deregulated transcripts under these conditions. *Sprouty4* is known as an inhibitor of the extracellular signal-regulated protein kinase (ERK/MAPK) pathway however its role in ES cells has not yet been defined. Gene expression and western-blot analyses have shown that Sprouty4 is highly expressed in ES cells and strongly downregulated upon differentiation whilst *in vivo*, Sprouty4 is confined to the founder population of ES cells, the inner cell mass of mouse blastocysts. Moreover, the *Sprouty4* promoter was found to be regulated via the direct binding of the intrinsic pluripotency-associated factors Nanog, Klf4 and Stat3. ES cells engineered to constitutively express a wild-type version of Sprouty4 were found to be resistant to differentiation induced by retinoic acid or embryoid bodies formation. Conversely, expression of a dominant negative Sprouty4 mutant activating the ERK/MAPK pathway in a sustained manner sensitized ES cells to differentiation and triggered endoreduplication leading to the formation of extraembryonic tissue.

Taken together, these results highlight the essential role of Sprouty4 in the tight regulation of the ERK/MAPK pathway- and probably others- for the balance between pluripotency and lineage commitment in mouse embryonic stem cells.

Keywords: Sprouty, MAPK, ES cells, pluripotency, differentiation

Zusammenfassung

Unbegrentzte Selbsterneuerungkapazität und Pluripotenz sind charakteristische Merkmale von embryonalen Stammzellen (ES-Zellen). Dennoch sind die molekularen und zellulären Mechanismen, die für das Schicksal der ES-Zellen zuständig sind, noch nicht genau definiert. Um regulierende Faktoren des undifferenzierten Zustands von ES-Zellen zu identifizieren, wurden undifferenzierte ES Zellen, „*Embryoid Bodies*“, spontan differenzierte und mit Retinsäure differenzierte ES Zellen mittels *Microarray*-Analysen verglichen.

Neben bereits etablierten Pluripotenz-Markern, wurde *Sprouty4* als eines der am stärksten degerulierten Transkripte unter diesen Bedingungen identifiziert. *Sprouty4* ist als Inhibitor des ERK (*Extracellular signal-regulated protein kinase*)-Signalweges bekannt, aber seine Rolle in ES-Zellen wurde noch nicht definiert. Mittels Genexpression und Western BlotAnalysen konnte gezeigt werden, dass Sprouty4 in undifferenzierten ES-Zellen stark exprimiert ist und im Verlauf der Differenzierung schnell herunterreguliert wird. *In vivo* war Sprouty4 auf die innere Zellmasse (*ICM*) der Mausblastozyste beschränkt. Außerdem wurde gezeigt, dass der *Sprouty4* Promotor durch direkte Bindung der PluripotenzMarkern Nanog, Klf4 und Stat3 reguliert wird. ES-Zellen, die Sprouty4 konstitutiv exprimieren, waren resistent gegen Differenzierung durch Zugabe von Retinsäure oder Bildung von Embryoid Bodies. Hingegen führte die Expression einer dominant-negativen Mutante von Sprouty4 zu einer erhörten Sensitivierung von ES-Zellen gegenüber der Differenzierung und zur Bildung extraembryonaler Gewebe begleitet von Endoreduplikation.

Zusammenfassend konnten unsere Ergebnisse zeigen, dass die enge Regulation des ERK-Signalweges und warscheinlich anderer Signalwege durch Sprouty4 notwendig ist, um die Balance zwichen Pluripotenz und Differenzierung embryonaler Stammzellen zu kontrollieren.

Schlagworte: Sprouty, MAPK, ES Zellen, Pluripotenz, Differenzierung

Contents

1 Introduction

Mammalian fertilization begins with the encounter and subsequent fusion of the egg and sperm. The haploid nuclei of the two gametes come then together to form a new diploid organism called zygote[1]. A broad array of specialized cells has then to be specified from this single totipotent cell in order to generate an entire organism. Some pathological conditions such as birth malformations are thought to be caused by defects occurring somewhere in this process[2]. Therefore, the ability to derive multiple lineages from embryonic stem (ES) cells is of great interest in order to understand how the earliest stages of lineage specification are regulated in normal embryonic development. Moreover, as the number of people needing transplants to restore tissue function after disease or injury far exceeds the number of compatible organs available for transplantation, a great hope is also placed on ES cells as an unlimited source of cells for the treatment of a myriad of diseases including diabetes[3], myocardial infarction[4], Alzheimer's and Parkinson's diseases[5]. In order to achieve all these purposes, it is however crucial to delineate the precise molecular mechanisms underlying the undifferentiated state of ES cells.

1.1 Origins and properties of embryonic stem cells

1.1.1 Teratocarcinomas and embryonal carcinoma

In 1970, two independent groups reported that normal early mouse embryos grafted into adult mice could induce the formation of tumorous growths known as teratocarcinomas[6,7]. These malignancies contained a variety of differentiated tissues such as skin and bone, mixed with a population of undifferentiated cells called embryonal carcinoma (EC)[8,9]. Only the grafting of pregastrulation embryos (before embryonic day E6.5) could generate teratocarcinomas and EC cells[10] (Figure 1). The vast majority of the isolated EC cell lines showed limited differentiation capacity both *in vitro* and *in vivo*[11]. Moreover, EC cells were almost always found to be aneuploid (abnormal number of chromosomes) and therefore unable to produce mature gametes. Yet, the study of EC cells strengthened the concept that pluripotent embryonic stem cells, i.e. cells that can develop into all cell types of the body apart from the extra-embryonic tissues, may be isolated from mammalian embryos.

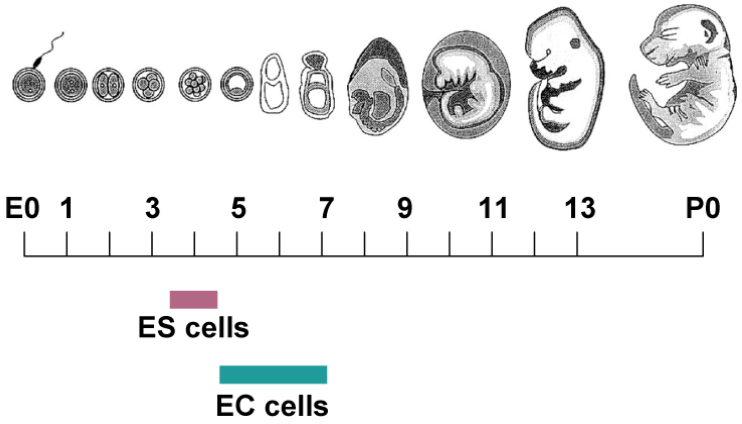


Figure 1: Developmental origin of murine embryonic stem cell lines (adapted from Wobus and Boheler, 2005).

The scheme illustrates the derivation of embryonic stem (ES) cells and embryonic carcinoma (EC) cells from different embryonic stages of the mouse. Pluripotent ES cells are established from the inner cell mass of the embryo between E3.5 and E4.5. EC cells are derived from malignant teratocarcinomas that originate from pregastrulation embryos (<E6.5) transplanted to extrauterine sites. Abbreviations: E, Embryonic day, P, Postnatal day.

1.1.2 Derivation of mouse embryonic stem cells

The observation that the pluripotency potential of EC cells could be enhanced by either co-culture with differentiated cell types or with medium conditioned with these same cells was crucial in the search for embryonic stem cells. Several potential "supporting" cell lines were investigated and mitotically inactivated embryonic fibroblasts (MEFs) were eventually found to be able to sustain the culture of EC cells that retain a high differentiation capacity[9,12]. MEFs were thought to secrete one or several critical factors and were thus described as feeder cells. The co-culture with MEFs on the one hand, and the use of conditioned medium on the other hand, enabled in 1981 the establishment of the first embryonic pluripotent cell lines directly derived from mouse blastocysts[13,14]. In the absence of feeder cells or conditioned medium, the self-renewing and pluripotent capacity of ES cells was lost, leading to preferential differentiation into neuronal tissues.

Pluripotent ES cell lines could be established from the inner cell mass of the embryo, i.e. the cells that will give rise to the embryo proper, between embryonic day E3.5 and E4.5[15]. ES cells closely resembled EC cells in morphology, growth behavior and marker expression but also in their capacity to give rise to teratocarcinomas[16]. However, in contrast to EC cells, ES cells were found to be able to integrate with high efficiency into a developing embryo following blastocyst injection and could contribute to a high rate of chimaerism in the fetal tissues[17]. Moreover, ES cells could efficiently colonize the germ cell lineage in a chimera leading to the formation of functional gametes[17].

1.1.3 Properties of mouse embryonic stem cells

1.1.3.1 Unlimited proliferation capacity and chromosomal stability

ES cells possess unique properties that distinguish them from any other cell types. One of these important features is that ES cells can be maintained and expanded in culture as a pure population of undifferentiated cells as judged by morphology and expression of a range of markers for extended periods of time, hypothetically indefinitely[18]. This property relates specifically to ES cells in culture and not to their embryonic counterparts, which do not remain as a stable stem cell population but differentiate into the three germ layers[19]. The efficient generation of equally potent subclones confirmed that ES cells undergo symmetrical self-renewal[11].

In somatic cells, the DNA complexes capping the ends of chromosomes known as telomeres are shorten with each cell division because DNA polymerases cannot replicate the distal end of the lagging chromosome strand[20]. This "end replication problem" eventually leads to a critical telomere length and cells either senesce or enter apoptosis[21]. Unlike somatic cells, ES cells can overcome the problem of critical telomere shortening by expressing the holoenzyme telomerase, a ribonucleoprotein complex which functions to extend and stabilize telomeric DNA, leading to increased chromosomal stability[22]. This ability to maintain a diploid karyotype even following extensive culture passaging partly explains the fact that ES cells appear to be immortal and show no evidence of senescence (i.e. cellular aging leading to growth arrest) over time in contrast to other primary cultures[23]. However, low-passage cells are preferably used and new subclones are regularly isolated in order to avoid random mutational events or epigenetic modifications such as loss of genomic imprinting (a form of gene regulation that results in only the copy inherited from the mother or father to function)[23].

1.1.3.2 An atypical cell cycle with a truncated G1 phase and no G1 checkpoint

The cell cycle consists of four distinct phases dedicated to the replication and transmission of genetic material to daughter cells. S phase and M phase are periods where chromosome replication and chromosome transmission occur, respectively; while G_1 and G_2 represent gap phases, which temporally separate S from M phase[19]. Murine ES cells have been shown to proliferate at unusually rapid rates with an atypical short generation time of approximately 8-10h in contrast to 16-24h normally[24]. Moreover, they have an uncommon cell cycle struc-

ture, consisting largely of cells in S phase and a truncated G_1 phase which lasts roughly 1.5h instead of approximately 8-10h[25]. Since most cell types spend the majority of their time in G_1 and since the S phase is similar within cell types, the short G_1 phase of mouse ES cells could account for their rapid cell division rate[19].

A restriction checkpoint is generally present during the G_1 phase to ensure that the DNA is intact and that the cell is functioning properly prior to chromosome replication. Functionally, safeguard proteins exist known as cyclin-dependent kinases (Cdk) consisting of a catalytic Cdk-subunit and a regulatory subunit known as cyclin[19]. In somatic cells, the G_1-S transition is regulated by cyclin D-Cdk4,6 and cyclin E-Cdk2 in early and late G_1 respectively[26,27]. In mouse ES cells, the cyclin D-Cdk4,6 complex is absent, whereas the cyclin E-Cdk2 complex is constitutively active[28]. Moreover, low activity of pocket proteins such as p107 and pRb leads to the constitutive activation of E2F target genes that are normally required for entry into S phase[24]. Therefore, ES cells lack the normal G_1 checkpoint that regulates the G_1-S transition. Upon ES cell differentiation, full gap phases are constituted, cell proliferation is slowed down and cell cycle-regulated Cdk activities are established[19].

1.1.3.3 Pluripotency

Similarly to their embryonic *in vivo* counterpart, ES cells have the potential to differentiate into cells representing all three germ layers of the embryo (ectoderm, mesoderm and endoderm) and are thus called pluripotent[11] (Figure 2). Even though ES cells can give rise to germ cells, they cannot produce all extraembryonic lineages and are therefore inaccurately described as totipotent[18].

ES cells can readily differentiate in monolayer culture leading to the expression of markers for mesoderm and endoderm[11]. However, the principal method used to trigger differentiation of ES cells into defined cell types is cell aggregation in suspension culture, which induces the formation of multi-differentiated structures called "embryoid bodies"[9,12]. Cellular differentiation proceeds on a schedule comparable to that in the embryo but without proper axial organization or elaboration of a body plan[29]. Each embryoid body has the potential to develop into multiple cell types and further differentiation is elaborated by subsequent attachment and outgrowth. The addition of retinoic acid can bias the differentiation towards certain cell types[30]. A range of differentiated tissues can be obtained from mouse ES cells *in vitro* including smooth muscle[31], cardiomyocytes[29], adipocytes[32] and neurons[33,34].

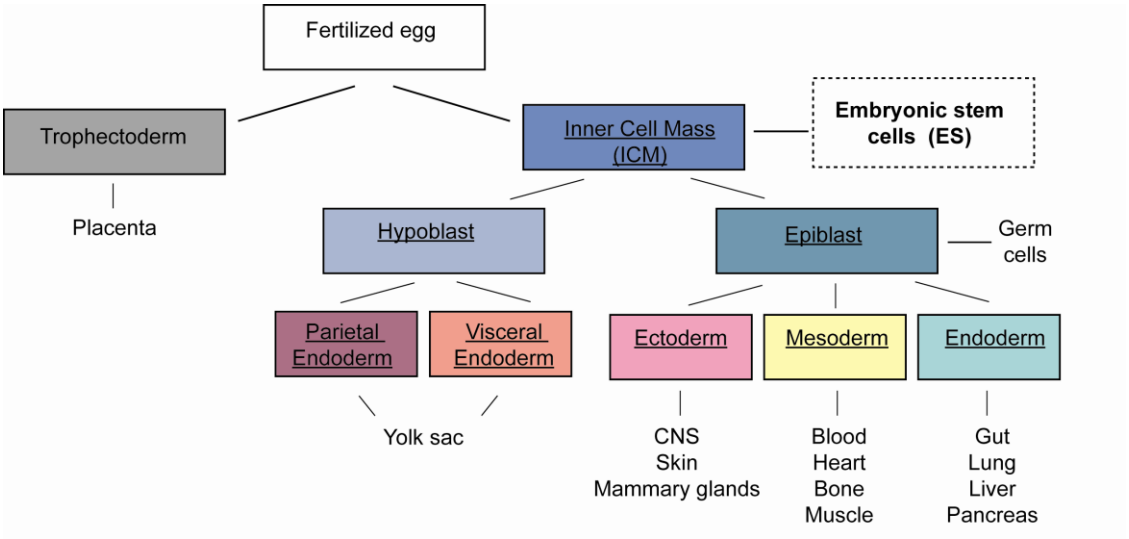


Figure 2: Scheme depicting ES cells in context of mouse development (adapted from Keller, 2005 and Smith, 2001).

Pluripotent ES cell lines are established from the inner cell mass of the embryo at the blastocyst stage between embryonic day E3.5 and E4.5. Lineages colonized by ES cells are underscored. ES cells rarely produce hypoblast derivatives in vivo but can do so in vitro. ES cells can differentiate into cells representing all three germ layers of the embryo (ectoderm, mesoderm and endoderm) but cannot produce all extraembryonic tissues.

1.1.4 Human embryonic stem cells

The dual capacities of mouse ES cells for unlimited expansion and for multi-lineage differentiation have naturally provoked high interest in establishing similar embryo-derived cell lines of human origin. The isolation of ES cells from non-human primates[35] in 1998 was the initial step towards the derivation of the first human ES cell lines from preimplantation embryos produced by in vitro fertilization[36,37,38].

Human ES cells share some fundamental characteristics to murine ES lines, such as the high proliferative potential, the formation of teratomas and basic cell cycle control mechanisms[11,19]. Nevertheless, distinctions exist between human and mouse ES cells based on cell surface marker expression and dependence upon specific self-renewal pathways (e.g. LIF, see below)[39,40], which suggests that these ES cell populations may not be interchangeable. The disparities observed between mouse and human ES cells may be attributed to the fact that ES cells exist only *in vitro* and may not necessarily recapitulate what is occurring in the embryo as cells had to adapt to culture conditions. Furthermore, the genetic heterogeneity of human ES cells may have an important impact on the disparities observed[11]. Recently, pluripotent stem cells sharing the unique properties of human ES cells have been derived from the late epiblast layer of post-implantation mouse embryos[41]. The authors suggested that human embryonic stem cells are an *in vitro* counterpart of human epiblast development and that

mouse and human ES cells are different because the latter ones represent a later developmental stage[41]. Altogether, this led to the hypothesis that the distinctions between mouse and human embryonic stem cells may not simply reflect species differences but rather diverse temporal origins[42].

As the focus of my research dealt mainly on murine ES cells, the subsequent chapters will essentially describe the signaling pathways which are important for the pluripotency of mouse ES cells.

1.2 Extrinsic regulators of pluripotency in mouse embryonic stem cells

1.2.1 The LIF/STAT3 signaling pathway

The sustained culture of ES cells in an undifferentiated state is not cell-autonomous[18] and the presence of all necessary metabolites and nutrients in the medium are not sufficient to support the growth of ES cells[18]. While co-culture with feeder cells was initially thought to be essential, it was later discovered that feeders could be substituted by conditioned medium[43]. This subsequently led to the identification of a critical cytokine called leukemia inhibitory factor (LIF; also known as differentiation inhibitory activity, DIA) which could sustain ES cell self-renewal in the absence of feeders[44,45]. *Lif*-deficient feeders are not able to support ES cell propagation[46]. Upon LIF or feeders removal, ES cells continue to proliferate but start to differentiate[47]. They lose their tight round colony morphology and progressively proliferate at a slower rate[47] (Figure 3). Interestingly, LIF is only able to sustain ES cells in the presence of serum, suggesting that additional factors are required[48]. *In vivo* studies showed however that the LIF cascade is not required for pregastrulation mouse development[15]. Indeed, mouse embryos lacking *Lif* can develop to a stage subsequent to ES cell derivation. This suggests that alternate pathways might be involved *in vivo* and *in vitro* for the maintenance of pluripotency.

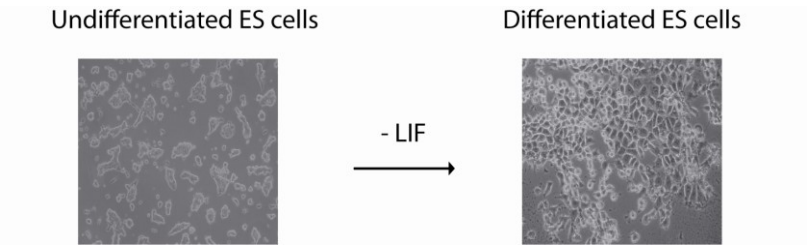


Figure 3: The effect of LIF on ES cell fate (adapted from Smith, 2001).

Photographs of self-renewing undifferentiated ES cells cultured in the presence of LIF (left) and differentiated ES cells three days after LIF removal (right). Abbreviation: LIF, Leukemia inhibitory factor.

LIF stimulates ES cells through gp130, the common signal transducer of all IL-6 cytokines, which works as a heterodimer together with LIFR, the specific receptor of LIF[49] (Figure 4). Activation of the gp130/LIFR receptor complex induces the Janus-associated tyrosine kinases (JAKs) to phosphorylate the receptor on its tyrosine residues. This leads to the recruitment and activation of the signal transducer and activator of transcription (STAT) family of transcription factors[50]. Phosphorylation of STATs promotes their dimerization, subsequent translocation to the nucleus and their binding to DNA target sites[50]. In ES cells, LIF predominantly activates STAT3[48].

Recruitment and activation of STAT3 was shown to be essential for self-renewal of ES cells[48,51] and overexpression of a STAT3 dominant-negative mutant abrogated the responsiveness to LIF and led to ES cell differentiation[25, 27]. A conditionally active form of STAT3 that can be induced by tamoxifen (a STAT3-Estrogen receptor (ER) fusion protein) was able to maintain the pluripotent phenotype of ES cells even in the absence of LIF[52]. However, it was later shown that the additional presence of serum contributed to self-renewal signals, establishing that STAT3 is not strictly sufficient for self-renewal[53].

Neither the LIF cytokine family nor STAT3 signaling are dedicated to stem cell regulation[11]. In actual fact, they have various effects on several cell types. Most of these actions are to promote differentiation, for instance of myeloid cells or astrocyte precursors, or to induce expression of differentiated functions such as acute phase protein synthesis by hepatocytes[54]. Therefore, ES cell self-renewal is stimulated by classical signaling transduction pathways but the result of this signal, that is inhibition of differentiation, is unique in the stem cell context.

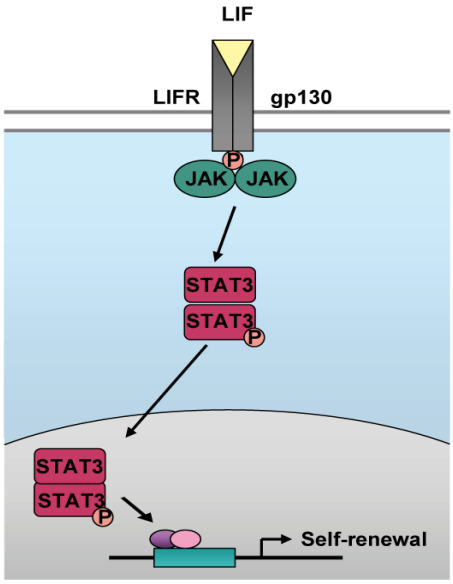


Figure 4: The LIF/STAT3 pathway (adapted from Stewart et al., 2006).
The cytokine LIF (Leukemia inhibitory factor) binds to its receptor (LIFR) at the cell surface, triggering it to heterodimerize with another transmembrane protein glycoprotein-130 (gp130). This leads to the recruitment of the non-receptor Janus-associated tyrosine kinase (JAK) which can phosphorylate the signal transducer and activator or transcription 3 (STAT3) triggering its dimerization and subsequent translocation to the nucleus. Binding of STAT3 to DNA target sites induces transcription of self-renewal genes.

1.2.2 The ERK/MAPK signaling pathway

Signaling downstream of gp130/LIFR is not limited to activation of STAT3 but includes stimulation of the Mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway regulates many different cellular responses in somatic cells including proliferation and differentiation[50].
In its simple form, the MAPK pathway can be directly activated upon binding of ligands that triggers the activation of receptor tyrosine kinases and other cell-surface receptors[55] (Figure 5). The growth factor receptor-bound protein 2 (Grb2) adaptor protein can bind through its SH2 domain to tyrosine phosphate docking sites located on the receptors themselves or on receptor substrate proteins, and through its SH3 domains to proline-rich motifs on the guanine-nucleotide-exchange factor Sos (son of sevenless), therefore triggering the formation of a Grb2/Sos complex on activated receptors[55]. Localization of Sos at the membrane promotes activation of the protooncogene Ras by inducing the exchange of GDP with GTP, which converts Ras into its active conformation[56]. Activated Ras functions as an adapter that binds to the serine/threonine-specific kinase Raf (or MAP-Kinase-Kinase-Kinase, MAPKKK)[57]

with high affinity and causes its translocation to the cell membrane, where a cascade of trans-phosphorylations is initiated. In mammals, three Raf proteins exist: Raf1, A-Raf and B-Raf[55]. Since Raf1 is ubiquitously expressed while A-Raf and B-Raf exhibit more restricted expression profiles, most of the studies concerning Raf actually refer to Raf1[55]. Raf then phosphorylates and activates MEK (MAP-Kinase-Kinase, MAPKK; also known as MAPK/ERK kinase) and the pathway finally culminates in the activation of extracellular signal-regulated protein kinases ERK1 and ERK2 (MAP-Kinase, MAPK; also called p44 and p42 respectively or ERK)[58]. Active ERKs phosphorylate cytoplasmic targets and also undergo nuclear translocation in order to modulate the activities of transcription factors such as Elk, Myc and the serum response factor SRF[59].

The recruitment of the Grb2/Sos complex can occur via indirect mechanisms as well. In the LIF pathway, the phosphotyrosines of the activated gp130/LIFR receptor serve as docking sites for the widely expressed tyrosine phosphatase SH2 domain-containing protein tyrosine phosphatase-2 (Shp2). The subsequent phosphorylation of Shp2 in turn recruits Grb2 and the scaffold protein Grb2-associated binder protein 1 (Gab1) therefore potentiating the coupling to the ERK/MAPK pathway[60].

The ERK/MAPK pathway can also be activated in a Ras-independent manner. The binding of growth factors such as VEGF (vascular endothelial growth factor) can activate a receptor tyrosine kinase signaling pathway mediated by phospholipase Cγ (PLCγ), which hydrolyzes phosphatydilinositol (4,5)-biphosphate (PIP_2) to form two second messengers: inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG)[61]. IP_3 activates its receptor on the endoplasmic reticulum leading to the mobilization of intracellular calcium (Ca^{2+}). Both DAG and Ca^{2+} activate Serine/Threonine kinases of the Protein kinase C (PKC) family including PKCδ which can phosphorylate Raf, resulting finally in the activation of the ERK/MAPK pathway independently of Ras[62].

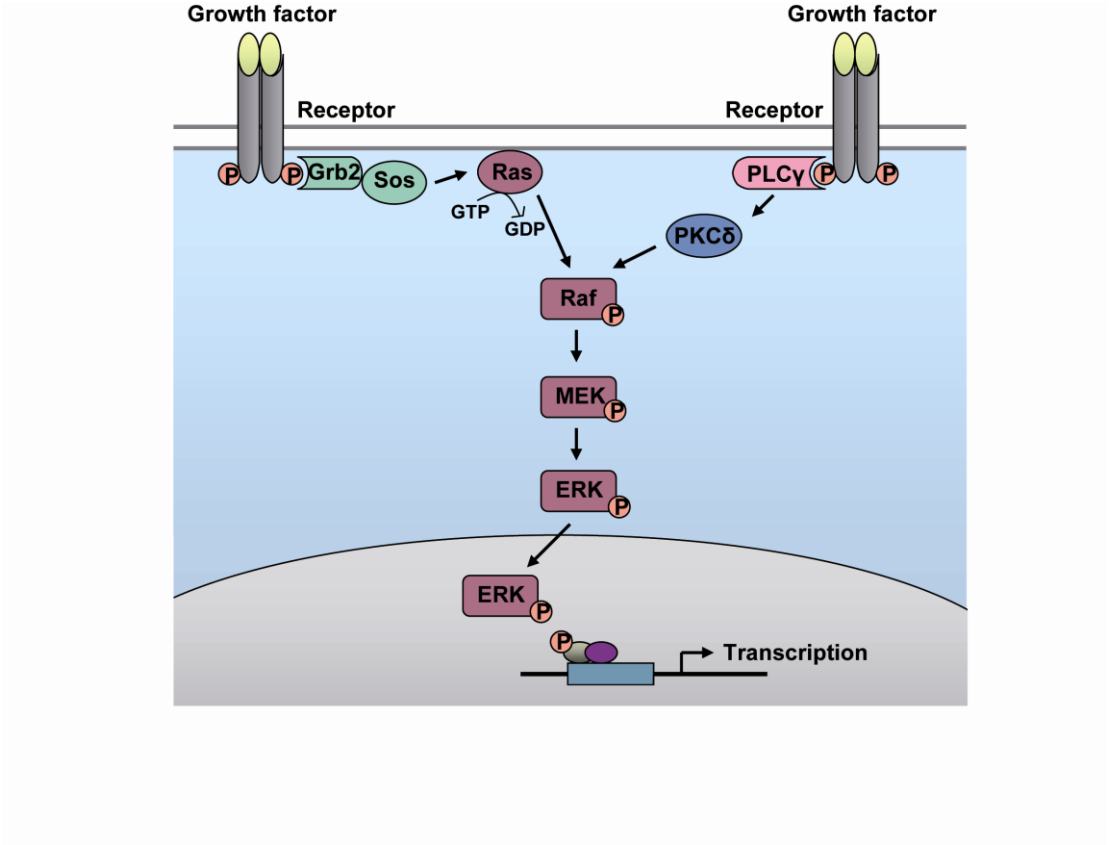


Figure 5: The ERK/MAPK pathway (adapted from Kim and Bar-Sagi, 2004).
Growth factor stimulation leads to receptor dimerization, tyrosine autophosphorylation and activation of intrinsic protein tyrosine kinase activity. Subsequently, the complex constituted of growth factor receptor-bound protein 2 (Grb2) adaptor protein and the guanine-nucleotide-exchange factor Sos (son of sevenless) is recruited. Sos then activates the small GTPase Ras, which in turn stimulates a phosphorylation cascade involving Raf (MAP-Kinase-Kinase-Kinase, MAPKKK), MEK (MAP-Kinase-Kinase, MAPKK), and ERK (MAP-Kinase, MAPK or Extracellular signal-regulated kinase). The phosphorylated ERK translocates to the nucleus where it phosphorylates and activates transcription factors such as c-Myc and Elk that control the expression of several target genes. Binding of growth factors such as vascular endothelial growth factor (VEGF) leads to the recruitment of phospholipase Cγ (PLCγ) to the phosphorylated receptor, which activates the protein kinase Cδ (PKCδ). PKCδ, in turn, phosphorylates and activates Raf, potentiating the coupling to the ERK pathway.

1.2.2.1 Regulation of ERK/MAPK signaling by Sprouty proteins

Since the MAPK-mediated signaling cascade governs a wide variety of processes including proliferation, differentiation, migration and survival, its activity is tightly controlled[63]. The signal transduction cascade is spatio-temporally regulated by balanced action of both positive and negative modulators that function at multiple levels of the signaling pathway and at different time points within the growth factor-induced response[63,64,65] . If this process is unhinged, severe developmental defects or tumors can arise[63].

1.2.2.1.1 The Sprouty family of proteins

Sprouty (Spry) proteins represent a major class of ligand-inducible inhibitors of receptor tyrosine kinases-dependent signaling cascades. They were originally identified in *Drosophila melanogaster* in genetic screens for regulators of tracheal[66] and eye[67] development. *Drosophila* Sprouty (dSprouty) has been shown to inhibit signaling induced by various receptor tyrosine kinases including FGF (fibroblast growth factor), EGF (epithelial growth factor), Torso (*Drosophila* homolog of PDGF, platelet-derived growth factor) and Sevenless (*Drosophila* homolog of HGF, hepatocyte growth factor)[67,68,69] receptors, which consequently established dSprouty as a general inhibitor of Ras signaling. *Drosophila* Sprouty is a 63kDa protein with a unique cysteine-rich C-terminal domain[66]. Based on sequence homology, four orthologs (Sprouty1-4) were identified in the mouse, human, chicken, zebrafish and frog[66,70,71,72,73,74,75]. Recently, new distant members of the Sprouty family called Spred (Spred1-3) were described[76]. Spred proteins possess a C-terminal Sprouty-related domain and an N-terminal Enabled/VASP homology 1 domain (EVH1). Similarly to Sprouty, they are able to inhibit the ERK/MAPK signaling pathway after mitogenic stimulations. Vertebrate Sprouty proteins are much smaller than the *Drosophila* homolog, with predicted molecular sizes in the 32-36kDa range[62]. Homology to the fly protein however is mainly restricted to the cysteine-rich region termed the Sprouty domain (Figure 6). Of the 22 cysteine residues found in dSprouty, at least 18 are present in each vertebrate protein. The Sprouty2 protein exhibits the highest sequence conservation across species[62]. The Sprouty domain also contains a highly conserved motif that mediates binding to Raf (Raf-binding domain, RBD). The N-terminal region of the Sprouty proteins is more divergent except for the presence of an invariant tyrosine residue (Y)[62].

1.2.2.1.2 Sprouty mechanisms of action

In the case of Sprouty1 and 2, this highly conserved tyrosine residue has be shown to be phosphorylated upon EGF and FGF signaling, while for Sprouty4, it was phosphorylated upon FGF and insulin signaling[77,78,79,80]. It is believed that the Src family of kinases (Src) is responsible for the phosphorylation of Sprouty at this conserved tyrosine, whereas the tyrosine phosphatase Shp2 could be involved in the dephosphorylation[80,81,82,83]. Another characteristic of Sprouty proteins is their capacity to form homo- and heterocomplexes[77,80,84,85]. In the context of FGF signaling, all four Sprouty isoforms have been reported to interact with each other via their cysteine-rich domains[86]. Interestingly, it has been shown that the interaction between Sprouty1 and Sprouty4 was the most potent combination

for inhibiting FGF2-induced MAPK signaling[86]. Therefore, the combinatorial assortment of Sprouty isoforms within a cell might determine the amplitude of inhibition.

Figure 6: Schematic representation of Drosophila and mouse Sprouty proteins (adapted from Mason et al., 2006).

Abbreviations: Y, tyrosine residue; RBD, Raf-binding domain; Spry, Sprouty; aa, amino acids.

The expression of Sprouty1, 2 and 4 is relatively widespread while that of Sprouty3 is thought to be more restricted (brain and testes in adult tissues)[73,87]. Upon growth factor activation, a subset of Sprouty proteins translocate to the cell membrane where MAPK inhibition presumably occurs[88,89,90]. Sprouty proteins can modulate MAPK activation induced by various stimuli such as NGF (Neural growth factor), insulin, SCF/KL (stem cell factor/kit ligand), GDNF (glial-derived growth factor), BDNF (brain-derived neurotrophic factor), and T-cell receptor activation[62,77,87,91,92,93,94,95,96].

Sprouty proteins specifically inhibit the ERK signaling pathway by receptor tyrosine kinases. However, whether or not Sprouty proteins impinge on other pathways than ERK is still an open question. Some groups observed that Sprouty could inhibit the ERK pathway without affecting the phosphoinositide 3-kinase (PI3K) or other MAPK pathways[87,97] whereas others claimed that Sprouty2 can disrupt both ERK and PI3K pathways[98]. Moreover, Sprouty1 and Sprouty2 can inhibit FGF- and VEGF- induced ERK activation but fail to repress EGF-induced or Phorbol 12-myristate 13-acetate (PDBu)-induced ERK activation[88,99]. This suggests that in contrast to *Drosophila*, mammalian Sprouty proteins may be selective rather than general inhibitors of receptor tyrosine kinase.

The exact point at which Sprouty blocks the ERK pathway remains also controversial and it is probable that distinct mechanisms can be activated depending on the cellular context and/or the identity of the upstream growth factor receptor. Several studies have concluded that

Sprouty proteins act upstream of Ras since ERK activation induced by activated Ras could not be inhibited[85,87,99,100]. However other groups demonstrated that Sprouty was able to repress FGF-induced activation of Raf without affecting Ras[97,101]. Therefore, Sprouty proteins might be capable of inhibiting ERK activity through a Ras-independent manner.
A common mechanism that is used to tightly control important signaling pathway modulators such as Sprouty protein is the establishment of a negative-feedback loop. It is thought that Sprouty expression can be induced through the same signaling pathway that they regulate[62]. As a consequence, the expression pattern of mammalian Sprouty proteins during embryonic development greatly coincides with known sites of receptor tyrosine kinase signaling including the developing lung, brain, heart, gut and muscle[73,102]. Similarly, dSprouty expression and EGF activity are found to be interdependent during *Drosophila* embryonic development, most notably in the eye disc known as the wing imaginal disc[67,68,69]. The promoters of Sprouty1, 2 and 4 contain GC-rich regions that include putative binding sites for numerous transcription factors[103,104,105]. However, the factors that control the tissue-specific expression of these Sprouty genes remain unidentified. Interestingly, a 69-bp Ras-responsive element has been mapped in the Sprouty4 promoter[104]. Temporal restriction of Sprouty repressive activity may be crucial for many developmental processes in which modulation of signal strength and kinetics defines the induction of the correct physiological outcome. To that purpose, it has been speculated that Sprouty protein levels are controlled through complex formation with c-CBL, an E3 ubiquitin ligase and adaptor molecule that promotes polyubiquitylation and subsequent proteasomal degradation of Sprouty proteins[80,106,107]. Yet, unlike Sprouty1 and 2, no interaction could be observed between Sprouty4 and c-CBL[80,108]. Therefore, the requirement of CBL proteins for Sprouty function remains uncertain.

1.2.2.1.3 Sprouty animal models

Ectopic overexpression and gene-targeting studies have helped to partly unravel the physiological role of Sprouty proteins. Gain- and loss of function experiments in *Xenopus tropicalis* have shown that Sprouty proteins could regulate mesoderm formation and cell movement during gastrulation by respectively modulating ERK and calcium signaling pathways downstream of the FGF receptor[109]. In the mouse, a role for Sprouty2 in lung branching morphogenesis was demonstrated through antisense oligonucleotide[75] and overexpression strategies[75,110]. It was also observed that Sprouty2 and Sprouty1 decreased uteretic branching and kidney development, suggesting that Sprouty proteins may play a major role in tubular morphogenesis[105,111]. Furthermore, in a mouse model engineered to harbor a germline

oncogenic K-ras^{G12D} mutation, lung tumorigenesis was found to be associated with elevated levels of Sprouty2, suggesting a tumor suppressor role for Sprouty2[112]. These findings indicate that Sprouty proteins may play a critical role in the regulation of oncogenic K-ras and implicate counter-regulatory mechanisms in the pathogenesis of Ras-based disease.

1.2.2.2 Sprouty4

Since Sprouty4 was the focus of my project, the following subchapter will solely concentrate on this particular family member.

The fourth member of the Sprouty family is 300 amino acids long and possesses the conserved C-terminal cysteine-rich Sprouty domain (Figure 6). Importantly, the Sprouty domain of Sprouty4 also includes a Raf-binding domain called RBD between amino acids 151-222 that mediates binding to Raf[62]. Moreover, an invariant tyrosine residue at position 53 is present in the N-terminal region. The exact point at which Sprouty4 blocks the ERK pathway remains controversial as well (Figure 7).

In the mouse, Sprouty4 has been shown to inhibit VEGF-induced ERK activation through binding to Raf via its C-terminal RBD domain independently of Ras[101]. A dominant negative mutant form of Sprouty4 lacking the RBD domain could still form oligomers and localize to the membrane but reversed the inhibitory effect of wild-type Sprouty4[101]. This demonstrates that the binding of Sprouty4 to Raf is necessary for the inhibition of VEGF- but not EGF-induced signaling. In contrast, others found that human Sprouty4 could repress insulin and EGF-induced ERK signaling upstream of, or parallel to, Ras[113].

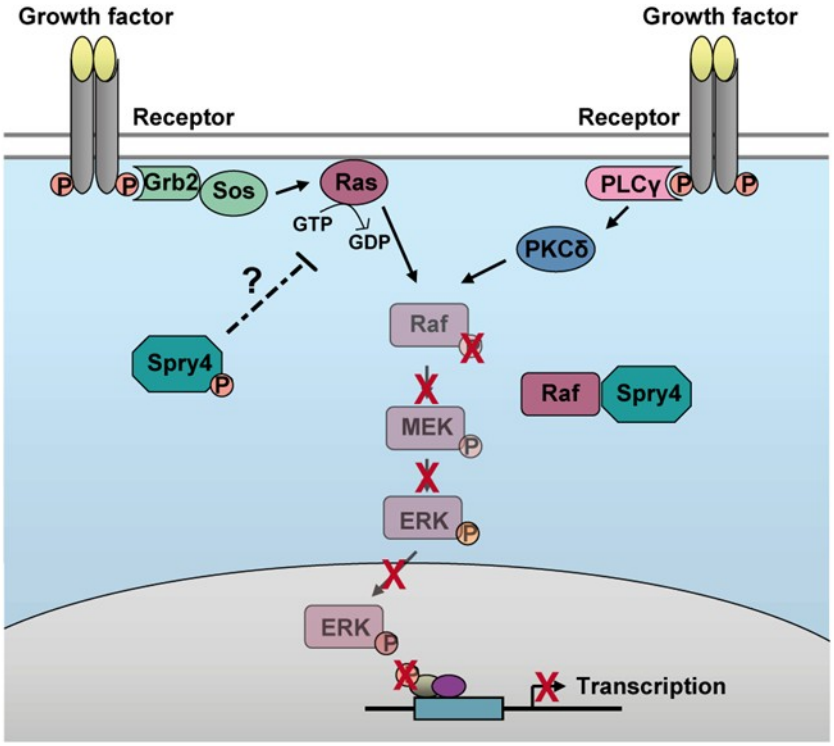


Figure 7: Repression of the ERK/MAPK pathway by Sprouty4 (adapted from Kim and Bar-Sagi, 2004 and Mason et al., 2006).

Sprouty4 can bind Raf via its C-terminal Raf-binding Domain (RBD) and this interaction inhibits the phosphorylation and activation of Raf, and subsequent activation of ERK. Sprouty4 might also repress the activation of ERK through a Ras-dependent mechanism. A Src-like kinase (Src) might phosphorylate Sprouty4 on its conserved tyrosine residue at position 53 (Y53) leading to the inhibition of Ras activation through a mechanism that is still unknown. An unidentified tyrosine phosphatase, potentially Shp2, might return Sprouty to a dephosphorylated state.

The mutation of the conserved tyrosine residue at position 53 in the N-terminal region revealed that tyrosine phosphorylation of Sprouty4 is necessary for suppressing Ras-dependent FGF- but not EGF-induced activation[77]. However, the mutation of this conserved tyrosine residue could not prevent the inhibition of Ras-independent VEGF signaling by Sprouty4[101]. The mechanism by which Sprouty4 can inhibit Ras is still undetermined although a report has suggested that the phosphorylation of the tyrosine residue could enable Sprouty protein to bind to Grb2, thereby inhibiting Ras activation[85].

Consistently, a direct interaction with Grb2 could be established for Sprouty1[85] and Sprouty2[85,114]. However, to date, Grb2 has not yet been shown as a binding partner of Sprouty4[62]. Another study in endothelial cells demonstrated that mouse Sprouty4 could interfere with FGF and VEGF signaling upstream of Ras since Sprouty4 inhibition could be overcome by a constitutively active form of Ras[99]. Overall, these results show that receptor tyrosine

kinases use distinct pathways for ERK activation and that Sprouty4 differentially regulates these pathways (Figure 8).

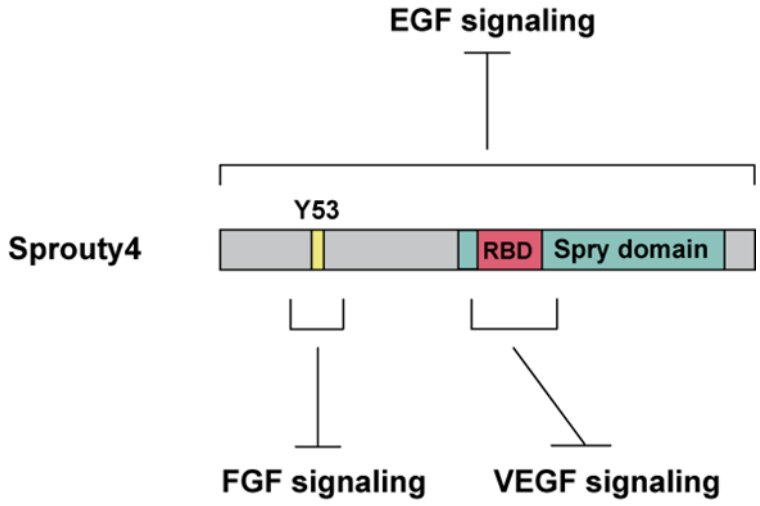


Figure 8: Selective inhibition of ERK signaling by the different domains of Sprouty4 (adapted from Sasaki et al., 2003).

Sprouty4 inhibits VEGF- but not EGF-induced ERK activation through binding to Raf via its C-terminal RBD domain independently of Ras[101]. The tyrosine phosphorylation of Sprouty4 is necessary for suppressing Ras-dependent FGF- but not EGF-induced activation[77]. The mutation of this conserved tyrosine residue could not prevent the inhibition of Ras-independent VEGF signaling by Sprouty4[101]. Abbreviations: VEGF, vascular endothelial growth factor; EGF, epithelial growth factor; FGF, fibroblast growth factor; RBD, Raf-binding domain.

In an attempt to elucidate the function of Sprouty4 *in viv*o, two independent groups have developed *Sprouty4* knockout (KO) mice[115,116]. One group used these mice to reveal that Sprouty2 and Sprouty4 control tooth development by repressing FGF signaling from different tissue compartments; Sprouty2 and Sprouty4 being expressed in the epithelium and mesenchyme respectively[115]. In a second report, *Sprouty4* KO mice showed reduced viability due to mandible defects, while the surviving mice exhibited growth retardation and polysyndactyly (i.e. fusion and duplication of forelimb digits)[116], thereby confirming previous gene-trap experiments in ES cells which suggested limb deformation[117]. In *Sprouty4* KO mouse embryonic fibroblasts, ERK activation was found to be sustained upon FGF stimulation, but not upon EGF[117]. In addition, *Sprouty4/Sprouty2* double KO mice were embryonic lethal with the majority of embryos dying at E12.5 presenting severe defects in craniofacial, limb, and lung morphogenesis[117]. These findings suggest both redundant and non-redundant functions for Sprouty4 and Sprouty2 on embryonic development and FGF signaling.

Although a role for Sprouty proteins in ES cells has not yet been reported, activation of the MAPK/ERK pathway has been shown to antagonize ES cell self-renewal.

1.2.2.3 Activation of MAPK signaling leads to embryonic stem cell differentiation

The use of a chimeric gp130 receptor with a mutated cytoplasmic domain preventing Shp2 association was shown to block coupling to the Ras pathway and enhance ES cell self-renewal[118]. Moreover, the growth of undifferentiated ES cells can be enhanced by culture in the presence of the MEK inhibitor PD098059, which further confirms that activation of ERK actively impairs self-renewal[118]. Differentiation can be hindered as well by genetic disruption of either *Grb2* or *Shp2*[119,120]. Knockout of *Grb2* led indeed to inhibition of primitive endoderm[119,121] and trophectoderm[122] formations. The reintroduction of a Grb2-Sos fusion protein or an activated form of Ras into *Grb2* null ES cells could restore normal differentiation[119]. Interestingly, *Erk1* null mice are viable and fertile[123] while disruption of *Erk2* is embryonic lethal due to defective placenta formation, trophectoderm and mesoderm differentiation[96,124]. Taken together, these results further demonstrate the role of the MAPK pathway in the early embryonic development and suggest that *Erk1* and *Erk2* have distinct biological functions. Therefore, in ES cells, the ERK pathway seems to promote differentiation and proliferation while antagonizing self-renewal.
The pluripotency of ES cells has been shown to depend not only on intracellular signaling pathways but also on a constellation of transcription factors involved in the development of the embryo[125].

1.3 Intrinsic regulators of pluripotency in mouse embryonic stem cells

1.3.1 Oct4

Oct4 (also known as *Oct3/4* or *Pou5f1*) encodes a homeodomain-containing protein called "Oct" because of its capacity to bind target genes through the "octamer" DNA sequence 5'-ATGCAAAT-3'[126]. Oct4 belongs to a family of transcription factors sharing a conserved DNA-binding domain, the POU-domain, which was originally identified in the transcription factors Pit1, Oct1 and Unc86[126]. The POU domain consists of two structurally independent subdomains: the POU-specific domain (POU_S) and the homeodomain (POU_H) which are connected by a flexible linker[126]. Interestingly, Oct4 was shown to either repress or activate

transcription of target genes depending on the flanking sequences[127]. Oct4 can also act on a subset of genes by binding to alternative A/T rich sites[128].

Expression of Oct4 occurs in the unfertilized egg and the early embryo prior to inner cell mass allocation[129]. Following the segregation of the blastocyst, Oct4 is downregulated in the trophectoderm while being strongly expressed in the inner cell mass. Expression of Oct4 is maintained in the epiblast of pre- and postimplantation embryos before becoming restricted to the migratory primordial germ cells where it persists through the formation of the genital ridges[130]. Oct4 is abundantly expressed in mouse ES and EC cell lines[131,132,133,134].

Oct4-deficient mouse embryos only develop to a stage that resembles a blastocyst but the cells that are allocated to the interior are only composed of trophectoderm cells[135]. These so-called blastocysts therefore lack a genuine inner cell mass[135]. *In vitro* outgrowths of *Oct4*$^{-/-}$ blastocysts led to the formation of colonies entirely consisting of trophectodermal cells[135]. The failure of *Oct4* mutant outgrowths to differentiate into hypoblast or definite germ layers indicates a severe defect in the potency of the cells allocated to the interior of the blastocyst in the absence of *Oct4*.

Conditional *Oct4* repression under the control of tetracycline in Oct4$^{+/-}$ ES cells induced loss of pluripotency and dedifferentiation of ES cells into trophectodermal cells[136]. Conversely, less than two-fold increase in expression of Oct4 triggered ES cell differentiation into hypoblast (primitive endoderm) and mesoderm[136]. Therefore, a critical amount of Oct4 is required to sustain ES cell self-renewal, and up- or downregulation ultimately determine cell fate. However at this stage, the target genes and the interacting partners of Oct4 are only partly known[128,137] (Figure 9). Although Oct4 has been for a long time considered as the sole master of pluripotency or stemness gene, i.e. a gene that confers stem-cell properties on a cell, it is becoming more and more evident that this crucial transcription factor is not sufficient on its own and that cooperation with interacting partners determines its effects on ES cell fate.

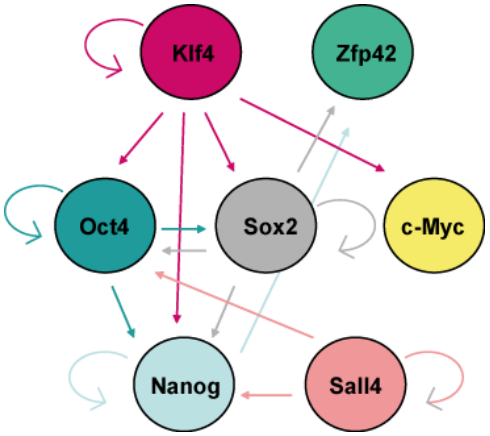


Figure 9: Schematic representation of transcriptional regulatory circuit in ES cells (adapted from Kim et al., 2008).

Arrowhead indicates the direction of transcriptional regulation. Klf4, Oct4, Sox2, Nanog and Sall4 have previously been shown to have autoregulatory mechanism. Other factors (not represented here) have been found to have a role in the establishment and preservation of pluripotency in ES cells suggesting the existence of a complex transcriptional network.

1.3.2 Sox2

Sox2 (SRY-related high-mobility group (HMG)-box protein 2) is a transcription factor containing a HMG DNA-binding domain that is implicated in the regulation of transcription and chromatin architecture[138]. Sox2 participates in the regulation of the inner cell mass and its derivative cells. It is strongly expressed in ES cells but also in neural stem cells[15]. Sox2 plays an essential role in the transcription of several Oct4 target genes such as *Fgf4* by forming a synergical ternary complex with either Oct4 itself or the ubiquitous Oct1 protein[139] (Figure 9). *Sox2* mutant embryos are present at E3.5 but lack Oct4-expressing cells by E6.0[140]. Outgrowths of *Sox2*$^{-/-}$ blastocysts do not sustain an epiblast component but in contrast to *Oct4*$^{-/-}$, generate both trophectodermal and primitive endodermal cells[140]. However, the situation is complicated as some maternal Sox2 proteins are persistent[140]. Altogether, this indicates that a combinatorial system requiring Oct4 and Sox2 specifies the first three cell lineages that arise soon after implantation.

1.3.3 Nanog

Nanog was first described as an NK-type homeobox gene under the name ENK (early embryo-specific NK, where NK represents NK2, a synonym of the *Drosophila* gene *ventral nervous system defective*) due to its homology with members of the NK gene family and was shown to be preferentially expressed in ES cells[141]. Shortly afterwards, two independent groups identified Nanog as an intrinsic determinant of pluripotency by using either functional

cDNA expression cloning or an *in silico* differential expression analysis[142,143]. They subsequently renamed it after "Tir Nan Og", the mythological Celtic land of the "ever young". Like Oct4, Nanog is a homeodomain-containing transcription factor. However, because the identity within the Nanog homeodomain to the homeodomains of other mouse proteins is at best 50%, it is considered as a divergent homeodomain protein[144].

Nanog is not expressed in the unfertilized egg and is first detected in the compacted morula ("little mulberry", 16-cell stage) mainly in cells in the interior of the embryo, the future inner cell mass[142]. High levels of Nanog mRNA persist in the inner cell mass of the early blastocyst while no expression is observed in the trophectoderm[142]. In later blastocysts, Nanog mRNA is further restricted to the epiblast and excluded from the primitive endoderm. Eventually, Nanog expression diminishes prior to implantation[142]. This expression pattern could indicate that a tight regulation of Nanog is important in order to avoid an uncontrolled expansion of pluripotent cells. After implantation, Nanog is expressed in a subset of epiblast cells but is rapidly downregulated upon entry into the primitive streak[145]. Undifferentiated mouse ES cells express Nanog[142], however upon LIF removal the physiological levels of Nanog are not sufficient to prevent ES cell differentiation[143].

Nanog null ES cells differentiate into extraembryonic endoderm lineages, which is consistent with the lack of primitive ectoderm in Nanog null embryos *in vivo*[142]. In the presence of a LIF receptor antagonist, Nanog overexpressing cells are still able to self-renew in contrast to control cells[142]. Moreover, these cells are able to contribute to a chimeric mouse after blastocyst injection, showing that the overexpression of Nanog enabled the maintenance of fully pluripotent ES cells in the absence of LIF[142]. However, ES cells overexpressing Nanog were resistant, but not completely refractory to differentiation following LIF withdrawal or all-trans retinoic acid treatment[142]. Therefore, the forced presence of Nanog seems to delay, rather than block, the differentiation of ES cells. Similarly to Oct4, Nanog has been proposed to regulate ES cell self-renewal through the transcriptional repression of genes that promote differentiation such as GATA-binding protein 6 (*Gata6*), a marker for endodermal tissue[146]. Nanog overexpression has no effect on Stat3 activation and Stat3 cannot activate Nanog expression[142]. There exists a negative feedback mechanism whereby Stat3 can induce the expression of Socs3, which then binds to gp130 and inactivates LIF signaling[147]. However, as Nanog overexpressing-ES cells can still self-renew in the presence of constitutive Socs3 expression, it is clear that Nanog is able to function independently of Stat3[130]. Being able to act separately, Nanog and Stat3 can nonetheless act synergistically in order to achieve maximal self-renewal efficiency[142].

Nanog function requires the presence of Oct4. Effectively, the consequences of *Oct4* deletion in ES cells could be rescued by the introduction of Oct4 cDNA[148] into the cells but not by transfection of Nanog cDNA[142]. In contrast, the examination of blastocysts derived from a $Nanog^{+/-}$ x $Nanog^{+/-}$ intercross revealed the presence of *Oct4* mRNA, therefore indicating that Nanog is not essential for Oct4 expression[142]. However, a sequence matching the consensus binding sites for Oct4 and Sox2 is found upstream from the transcription start site of the *Nanog* gene[142]. The two potential binding sites for Oct4 and Sox2 are juxtaposed relative to one another in a similar manner as they are on known Oct4/Sox2 targets such as *Utf1*, which suggests that Oct4 and Sox2 may regulate Nanog expression[142] (Figure 9). Besides this potential Oct4 binding, little is known about the regulation of the *Nanog* gene, except that the transcriptional activator and tumor suppressor p53 can bind to the *Nanog* promoter leading to the suppression of Nanog expression[149]. Loss of *p53* increases the susceptibility to testicular teratoma[150], which suggests that teratoma formation might be due to derepression of genes such as *Nano*g.

Recently, Chambers *et al.* have suggested that Nanog acts primarily in the construction of inner cell mass and germ cell states rather than in the housekeeping machinery of pluripotency[151]. In addition, they found that transient downregulation of Nanog predisposed cells towards differentiation but did not induce commitment. Permanent genetic deletion of Nanog did not prevent ES cells to self-renew indefinitely even though they were prone to differentiation. Moreover, expanded Nanog null cells could colonize embryonic germ layers and exhibited multilineage differentiation both in fetal and adult chimaeras. Thus, they proposed that Nanog's role is to stabilize ES cells in culture by resisting or reversing alternative gene expression states.

1.3.4 Other factors

Next to Oct4, Sox2 and Nanog, other factors important for pluripotency have been identified including Zfp42 (Zinc finger protein 42, also known as Rex1 for reduced expression 1), Sall4 (Sal-like 4) and Klf4 (gut-enriched Krüppel-like factor 4 or gKlf)[152,153,154] (Figure 9).

Klf4 belongs to the Krüppel-like factor (Klf) family of evolutionarily conserved zinc finger transcription factors that regulate numerous biological processes, including proliferation, differentiation, development and apoptosis[155]. The depletion of Klf4 using shRNA in mouse ES cells did not induce differentiation, suggesting that Klf4 is not required for the maintenance of undifferentiated state of ES cells[154,156]. However, the simultaneous depletion of

Klf2, Klf4 and Klf5 lead to ES cell differentiation, which demonstrates that the Krüppel-like factors are required for the self-renewal of ES cells and that they have redundant functions[154]. Chromatin immunoprecipitation coupled to microarray analyses revealed that these Klf proteins share many common targets with Nanog and that Klf2, Klf4 and Klf5 directly bind to the enhancer region of Nanog, similarly to Sall4 and Nanog itself[154].

An essential role for these factors in pluripotency has been recently confirmed by the successful reprogramming of somatic cells with specific genes. The combination of Oct4, Sox2, c-Myc and Klf4 has been shown to reprogram mouse embryonic and adult fibroblast cells to a pluripotent state[157,158,159]. These same four factors have also been proven capable of resetting human dermal fibroblasts[160,161], whereas others have showed that Oct4, Sox2 and Nanog together with Lin28 were sufficient to establish pluripotent cells from human somatic cells[162]. How these proteins cooperate to induce pluripotency remains unclear but it is likely that, aside from their direct functions in transcriptional regulation, they interact with chromatin remodeling factors and histone modifying enzymes to modulate chromatin conformation[163]. Recently, Marson *et al.*[164] have connected non-coding microRNA genes to the core transcriptional regulatory circuitry of ES cells, revealing a new layer of complexity in the regulatory network that determines stem cell fates. The intricate circuitry underlying ES cell self-renewal and pluripotency therefore needs to be further investigated in order to appreciate its entire scope.

1.4 Aim of the study

Although the requirements for maintaining mouse ES cells in a self-renewing pluripotent state are increasingly being defined, the precise molecular and cellular mechanisms by which pluripotency is supported remain largely unknown. The growth of undifferentiated ES cells requires a balance between survival, proliferation and self-renewal signals that are regulated by distinct signaling pathways.

Knowledge of the intricate mechanisms regulating ES cell pluripotency and differentiation potential is currently limited to a few signaling pathways (e.g., LIF, BMP and Wnt) together with regulatory factors (e.g., Oct4, Nanog and Sox2). Since it is likely that pluripotency is determined by a broad constellation of molecular factors, analysis of the cellular transcriptome may enable to define the molecular phenotype of stem cells and establish the determi-

nants of ES cell fate. The hypothesis is that some mRNAs may be essentially or more abundantly expressed in embryonic stem cells than in any other cell types and that comparisons among cell populations (undifferentiated vs. differentiated) would reveal these differences.

The main goal of this study was to identify novel ES cell pluripotency-associated factors. As it has been shown that the growth of pluripotent ES cells could be enhanced by culture in the presence of MAPK inhibitors, the significance of the presence of the ERK/MAPK inhibitor Sprouty4 in the microarray data set among well-defined stem cell regulators was examined.

It was first investigated whether Sprouty4 had in ES cells a similar repressive activity on the ERK/MAPK pathway than in other somatic cells. Next, it was then tested whether constitutive Sprouty4 expression in ES cells could enhance pluripotency or protect the cells against differentiation. Finally, sustained expression of a dominant negative form of Sprouty4 was used to assess whether the disruption of Sprouty4 activity could alter the self-renewal capacity of ES cells and if it had functional consequences on lineage commitment.

2 Results

2.1 Identification of novel ES cell pluripotency-associated factors

To date, only a few signaling pathways and regulatory factors have been described to be important for the pluripotent state of ES cell[40,163,165,166]. Because it is highly probable that pluripotency is determined by a complex combination of molecular factors, comparison of the cellular transcriptome between undifferentiated and differentiated ES cell populations may enable the identification of novel regulators of ES cell fate.

To that purpose, whole genomic transcriptional profiling of pluripotent and differentiated ES cells was performed. To circumvent bias induced by lineage specificity or variation in the differentiation protocols, I relied on the use of three differentiation methods (Figure 10A). The first differentiation method consisted in culturing the ES cells as adherent monolayers in the absence of the self-renewal signals provided by feeder cells or LIF, which subsequently leads to the commitment of ES cells to a neural cell fate[167]. To accomplish the second differentiation procedure, ES cells were cultured as aggregates in "mass culture"[29] on non-adherent culture dishes in the absence of feeders or LIF leading to the formation of three-dimensional round-shaped structures called embryoid bodies (EB). EBs consist of an outer layer of endoderm-like cells, an ectodermal "rim" and specified mesodermal cells and are therefore considered as a powerful *in vitro* model system for the study of early lineage determination and organogenesis in mammals[168]. In the third differentiation type, ES cells were cultured in monolayers in the absence of feeder cells and LIF but in the presence of retinoic acid (RA), which promotes the formation of neuroectodermal tissue[30,34,169].

Mouse R1 ES cells were thus collected in two separate experiments at zero and three days of differentiation after either LIF removal, aggregation into embryoid bodies, or treatment with retinoic acid and their mRNAs were amplified and hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 array containing 39000 probe sets. The data were normalized and analyzed using the BRB-ArrayTools software. Only transcripts with a change of at least two-fold in either direction from the median value and a p-value of the log-ratio variation greater than 0.01 were admitted. As illustrated in the Venn diagram of Figure 10B, some transcripts were significantly deregulated in only one of the three differentiated cell-types while others were common to two out of the three populations.

Finally, a total of 47 overlapping genes were found to be differentially expressed in all three types of differentiated ES cells compared to undifferentiated ES cells using the significance analysis of microarrays (SAM) software (Figure 10 and Table 1). The presence of several

well-defined stem cell-associated transcripts such as *Zfp42*, *Klf4*, *Sox2, Nr0b1* and *Essrb* validated the data and the approach[170,171,172].

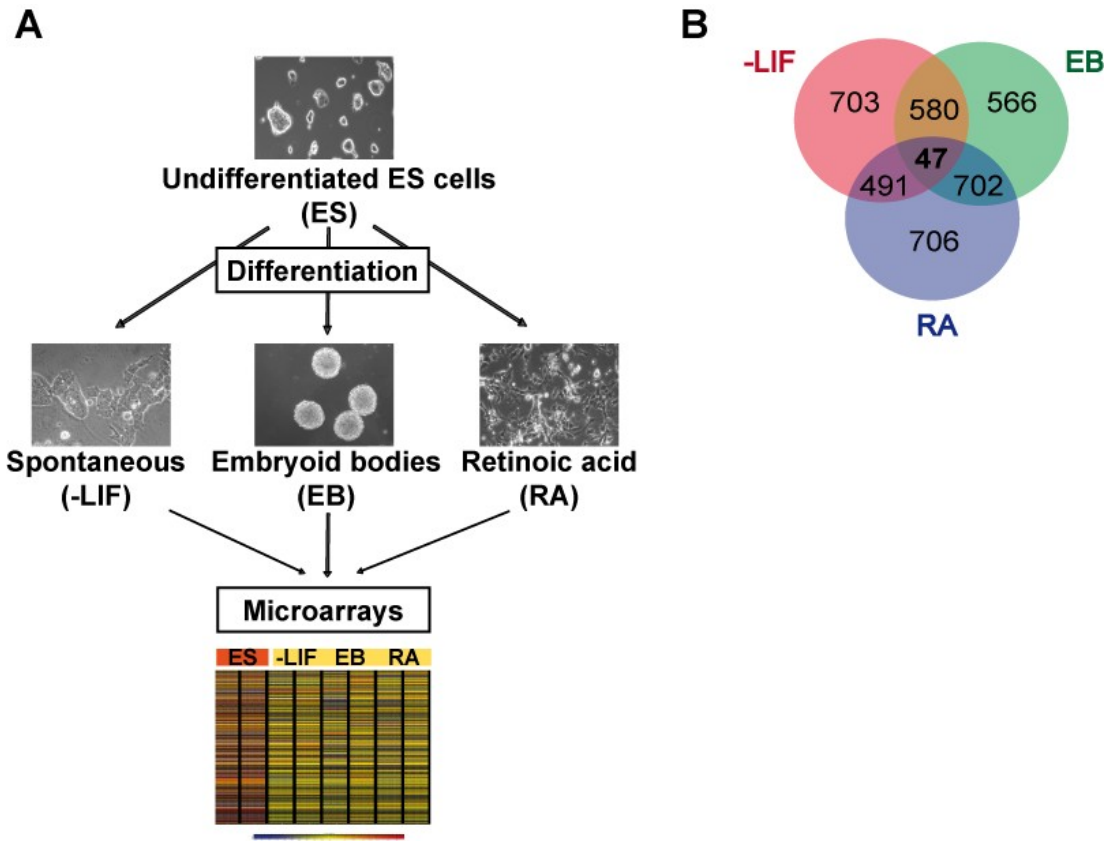


Figure 10: Pluripotency markers revealed by transcriptional profiling of mouse embryonic stem cells. **(A):** Mouse cDNA microarrays were used to compare total RNA from differentiated ES cells cultured in the absence of LIF (-LIF), grown into embryoid bodies (EB) or treated with retinoic acid (RA) with RNA from undifferentiated ES cells (ES). **(B):** Venn diagram illustrating the overlap of genes differentially expressed in all three differentiation protocols vs. undifferentiated ES cells.

Table 1: List of genes differentially expressed in all three types of differentiated ES cells (-LIF, EB, RA) compared to undifferentiated ES cells.

#	Gene symbol	Description	Fold change[a]	Probe set
	Nr0b1	nuclear receptor subfamily 0, group B, member 1	16.87	1417760_at
2	Zfp42	zinc finger protein 42	15.237	1418362_at
3	Klf4	Kruppel-like factor 4 (gut)	24.548	1417394_at
4	Jam2	junction adhesion molecule 2	15.775	1436568_at
5	Spry4	Sprouty homolog 4 (Drosophila)	14.69	1445669_at
6	Lrrc34	leucine rich repeat containing 34	11.789	1429366_at
7	Dnajc6	DnaJ (Hsp40) homolog, subfamily C, member 6	10.414	1433596_at
8	Nr5a2	nuclear receptor subfamily 5, group A, member 2	8.731	1420410_at
9		Transcribed locus	6.974	1456521_at
10	LOC433110	hypothetical LOC433110	13.012	1456242_at
11	Pcolce	procollagen C-endopeptidase enhancer protein	9.027	1437165_a_at
12	Klf4	Kruppel-like factor 4 (gut)	7.492	1417395_at
13	LOC545325	hypothetical protein LOC545325	7.575	1455604_at
14	Trim2	tripartite motif protein 2	9.324	1459860_x_at
15		Transcribed locus	7.7	1435374_at
16	Morc1	microrchidia 1	6.661	1419418_a_at
17	Tdh	L-threonine dehydrogenase	8.971	1449064_at
18	BC050188	cDNA sequence BC050188	7.069	1438872_at
19	Neurod1	neurogenic differentiation 1	6.839	1426412_at
20	Klf2	Kruppel-like factor 2 (lung)	6.081	1448890_at
21	Myst4	MYST histone acetyltransferase monocytic leukemia 4	6.568	1423508_at
22	Sgk	serum/glucocorticoid regulated kinase	7.893	1416041_at
23	Trim2	tripartite motif protein 2	6.053	1417027_at
24		PREDICTED: similar to PR domain containing 14	5.222	1444390_at
25	Spry4	sprouty homolog 4 (Drosophila)	5.101	1440867_at
26	Esrrb	estrogen related receptor, beta	5.617	1436926_at
27	Xist	inactive X specific transcripts	5.91	1436936_s_at
28	Cobl	cordon-bleu	6.476	1434917_at
29	E130014J05Rik	RIKEN cDNA E130014J05 gene	5.215	1455300_at
30	St8sia1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	5.333	1455695_at
31	BB001228	expressed sequence BB001228	6.244	1455425_at
32	Ttpa	tocopherol (alpha) transfer protein	5.045	1427284_a_at
33	Fgf4	Fibroblast growth factor 4 (Fgf4), mRNA	4.97	1449729_at
34	Epha4	Eph receptor A4	4.833	1429021_at
35		Transcribed locus	4.75	1447678_at
36	Sox2	SRY-box containing gene 2	5.756	1416967_at
37	Vegfc	vascular endothelial growth factor C	5.129	1439766_x_at
38	Jam2	junction adhesion molecule 2	6.771	1449408_at
39	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	5.426	1421840_at
40	D14Ertd668e	DNA segment, Chr 14, ERATO Doi 668, expressed	5.53	1438868_at
41	B230354K17Rik	RIKEN cDNA B230354K17 gene	4.647	1424515_at
42	Arid5b	Modulator recognition factor 2 (Mrf2)	5.454	1434283_at
43	Ddc	dopa decarboxylase	4.478	1426215_at
44	Spp1	secreted phosphoprotein 1	6.915	1449254_at
45	Ifitm1	interferon induced transmembrane protein 1	4.574	1424254_at
46	Fgf4	fibroblast growth factor 4	5.657	1420085_at
47	Vegfc	vascular endothelial growth factor C	4.43	1419417_at

Transcripts were analyzed using the significance analysis of microarrays (SAM). Fold change[a]: represents the fold difference of geometrical means of intensities.

To confirm the fidelity of the gene-array data, a set of 11 genes was selected and their transcript levels were tested by quantitative reverse transcription-PCR (qRT-PCR). The genes selected included some of the ES cells-associated markers such as *Zfp42* and *Klf4* as well as genes not yet linked to ES cell pluripotency (Figure 11). As shown by others[153,173], the expression levels of *Zfp42* and *Klf4* dramatically decreased upon differentiation achieving less than 5% of the initial expression in undifferentiated ES cells. Similarly to these ES cell markers, all the genes tested except two (*B230354K17Rik* and *Arid5b*) were found to be strongly downregulated in all three types of differentiated ES cells. The qRT-PCR analysis therefore revealed a high degree of correlation with the microarray data, providing confidence in the approach to identify differentiation-dependent genes in ES cells.

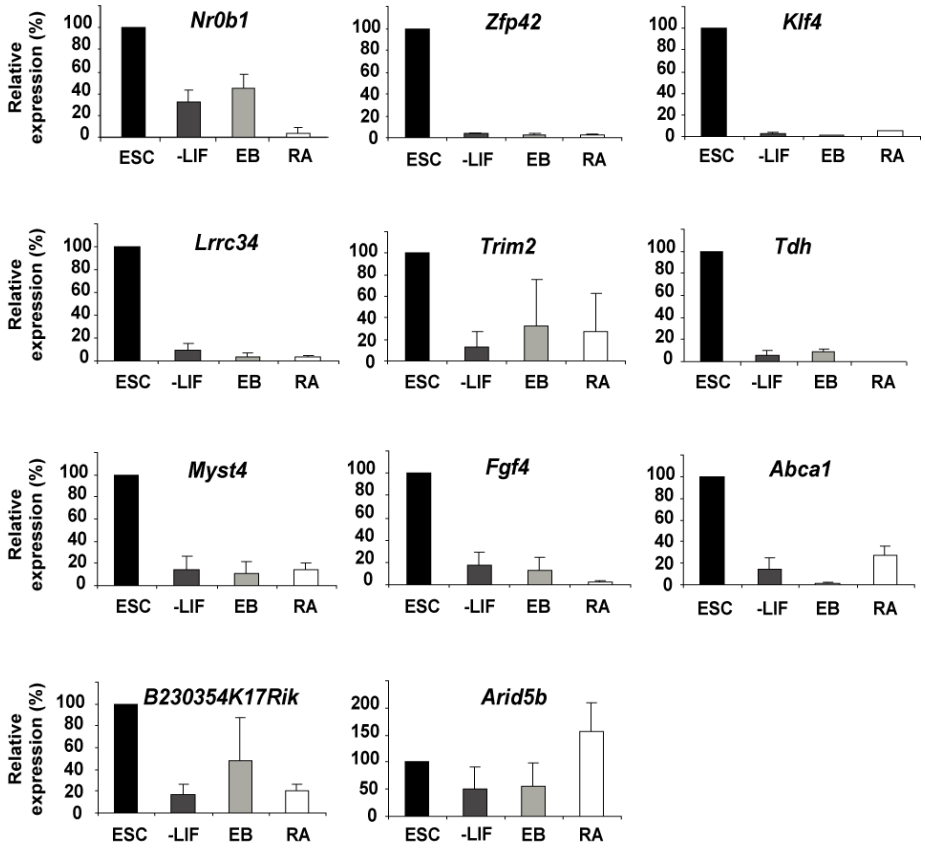


Figure 11: Verification of microarray results with quantitative RT-PCR.

The graphs display the relative expression of mRNA in differentiated ES cells cultured in the absence of LIF (-LIF), grown into embryoid bodies (EB) or treated with retinoic acid (RA) for two days compared to undifferentiated ES cells (ESC). All measurements were performed in duplicate in two separate experiments. The relative levels of gene expression of target mRNAs were normalized against Actin expression. Error bars represent the standard deviation.

2.2 Sprouty4 is highly expressed in ES cells and is dramatically reduced upon differentiation

The microarray analysis revealed one particularly interesting gene called *Sprouty4,* which was quickly repressed upon differentiation with a fold change in the same range as *Zfp42* and *Nr0b1* (14.69 vs. 15.24 and 16.87 respectively) (Table 1). The decrease in *Sprouty4* expression was verified by qRT-PCR. Figure 12A shows that Sprouty4 mRNA was strongly downregulated by at least 10-fold upon differentiation after either LIF removal, aggregation into embryoid bodies, or treatment with retinoic acid. To control the differentiation conditions, the same analysis was performed with *Oct4* and *Nanog,* two well-known pluripotency markers[15]. The expression pattern of *Sprouty4* upon differentiation was found to be similar to that observed for the two ES cell markers. Interestingly, *Sprouty4* downregulation seemed even more dramatic than the one observed for *Oct4* or *Nanog*. The microarray and qRT-PCR results have shown that Sprouty4 mRNA expression levels were significantly downregulated upon ES cell differentiation.

Next, the time-course expression pattern of Sprouty4 upon differentiation was assessed. ES cells were cultured in the absence of LIF as monolayers for nine days. Every three days the cells were trypsinized and one half was harvested while the other half was replated in the same culture conditions for a further three days. The qRT-PCR results in Figure 12B show that at the mRNA level, Sprouty4 expression greatly decreased after three days and even more after six days of differentiation upon LIF removal. After nine days, Sprouty4 expression increased slightly but was still much lower than in undifferentiated ES cells. Similarly, the pluripotent marker Oct4 progressively decreased after six days of differentiation and showed a small increase at day nine.

At the protein level, a strong band around 36kDa could be detected in ES cells cultured in the presence of LIF (Figure 12C). However, upon differentiation, Sprouty4 protein levels were found to gradually decrease until almost no protein could be detected at day nine. Sprouty4 has been shown to decrease the activation of ERK protein via the inhibition of the ERK/MAPK pathway[77]. Consistently, the active phosphorylated form of ERK showed an expression pattern inversely parallel to that of Sprouty4 and was increased progressively upon differentiation while the total ERK levels stayed constant. Taken together, these assays show that Sprouty4 has a differentiation-dependent expression pattern in ES cells both at the mRNA and protein levels, thereby substantiating the microarray results.

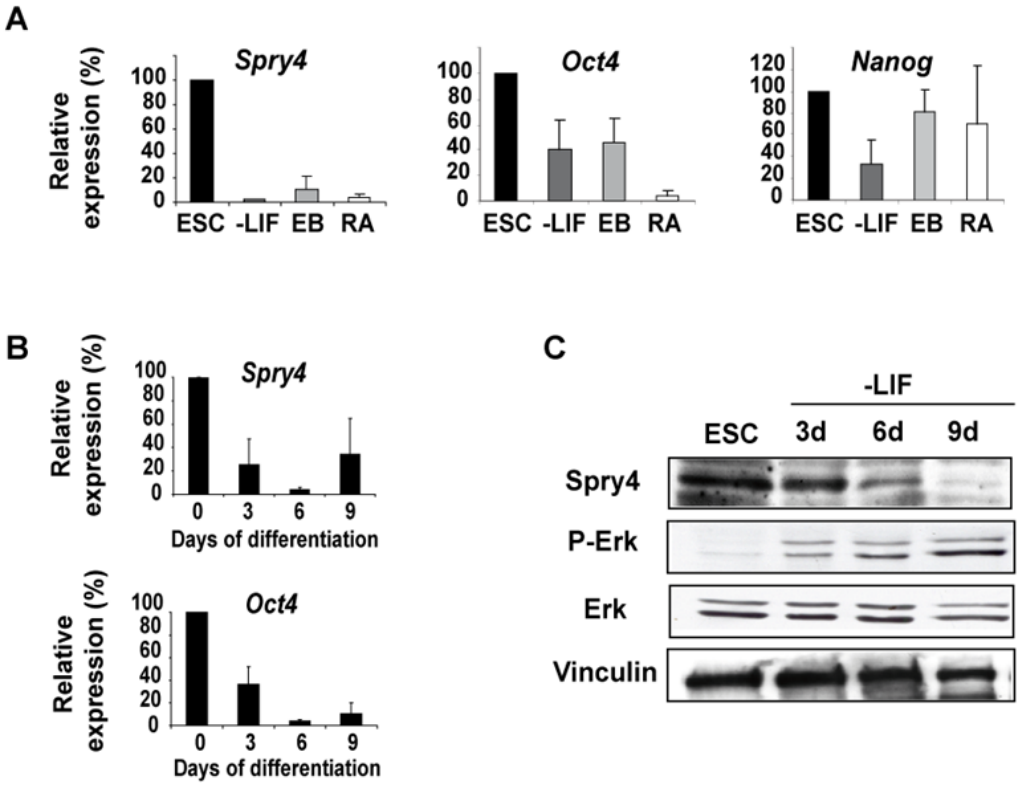


Figure 12: Expression of Sprouty4 decreases upon ES cells differentiation.
(A): Gene expression levels of pluripotency genes and Sprouty4 were determined by qRT-PCR in 3 days spontaneously differentiated ES cells (-LIF), embryoid bodies (EB) and RA-treated ES cells (RA). Data (representative of 3 separate experiments) are expressed as average expression levels ±SD relative to undifferentiated ES cells. **(B):** Gene expression levels of Spry4 and Oct4 determined by qRT-PCR in ES cells upon LIF removal for the times indicated. The relative expression is presented compared with undifferentiated ES cells at day 0. Oct4, a known pluripotency gene, was used as a control for the differentiation efficiency. **(C):** Western blots of Spry4, Phospho-Erk and total Erk in ES cells induced to differentiate in the absence of LIF for the number of days indicated. Vinculin was used as a loading control.

The western-blot shown in Figure 12C suggests that Sprouty4 is highly expressed in undifferentiated ES cells. It has been reported that Sprouty4 could be detected by Northern blot in the mouse embryo and in many adult tissues including heart, brain, lung, kidney and skeletal muscle[73]. Moreover, whole-mount *in situ* hybridization analysis showed that Sprouty4 was present in the mouse embryo at stages when organogenesis is commencing (E8.5-E9.5)[73]. At E8.5, Sprouty4 could be detected in the primitive streak, in the rostral forebrain and in cells lateral to the posterior hindbrain[73]. To further study Sprouty4 expression pattern in pluripotent ES cells, immunofluorescence microscopy was performed using primary antibodies directed against Sprouty4 on ES cells cultured in the presence of LIF (in collaboration with Dr. Tiziana Giordano, MDC Berlin). Oct4, a transcription factor known to be highly expressed in pluripotent ES cells[174], was taken as a positive control. These results revealed

that Sprouty4 was strongly expressed in undifferentiated ES cells cultured in the presence of LIF (Figure 13A). Furthermore, Sprouty4 could be detected in all the cells also positive for the pluripotency-associated factor Oct4. Interestingly, western-blot analysis using ES cell subcellular fractionation showed that Sprouty4 can be detected in both the nucleus and cytoplasm of undifferentiated ES cells since its expression coincided with the nuclear marker Lamin A and the cytoplasmic marker α-Tubulin (Figure 13B).

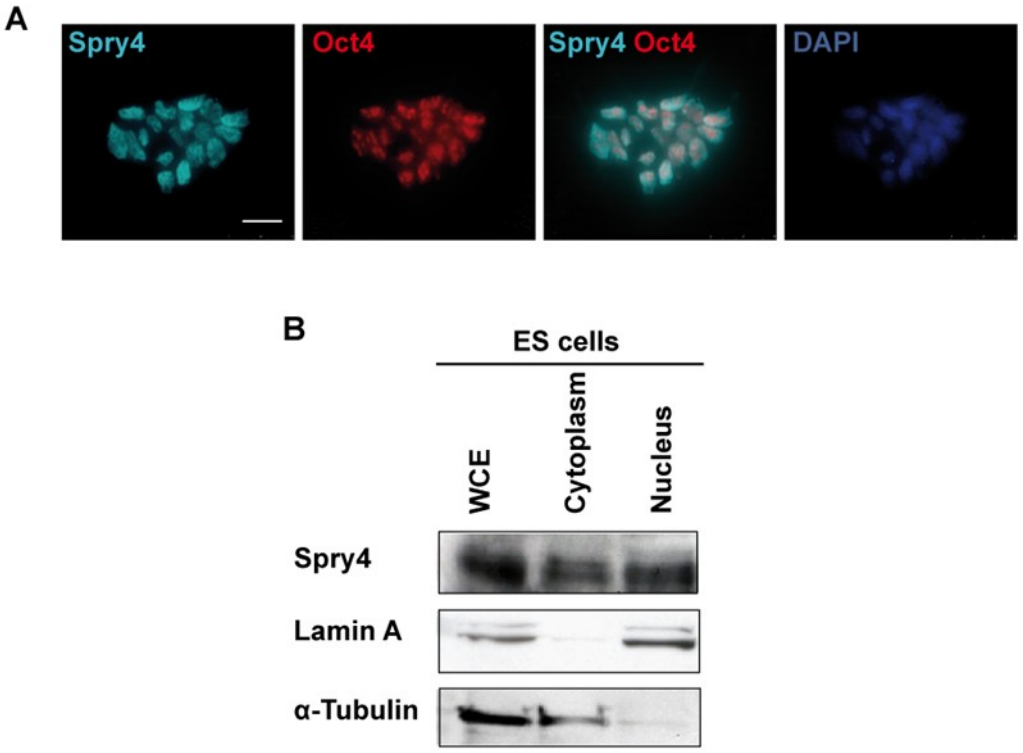


Figure 13: Sprouty4 localizes in the cytoplasm and nucleus of undifferentiated ES cells.
(A): Subcellular localization of Sprouty4 in ES cells was analyzed by immunostaining with a Spry4 antibody (cyan) and Oct4 antibody (red). 4, 6-Diamidino-2-phenylindole (DAPI) (blue) was used as a specific nuclear label. Scale bar: 25µm. **(B):** Cell fractionation of undifferentiated ES cells cultured in the presence of LIF were lysed and subjected to cell fractionation. Whole cell extract (WCE), cytoplasmic and nuclear fractions were analyzed by immunoblot using antibodies against Sprouty4, the nuclear marker Lamin A and the cytoplasmic marker α-Tubulin.

2.3 Sprouty4 is expressed *in vivo* in the inner cell mass of embryos

The results obtained by western-blot and immunofluorescence demonstrated that Sprouty4 is highly expressed in pluripotent ES cells. Since ES cells originate from the inner cell mass of blastocysts[11], distribution of Sprouty4 protein in the preimplantation mouse embryo was investigated *in vivo*. Previously, whole-mount in situ hybridization analyses have demonstrated that Sprouty4 could be detected in the mouse embryo at stages when organogenesis is start-

ing[73]. To date however, the expression pattern of Sprouty4 in the mouse embryo prior to E8.5 remains undescribed. Figure 14 shows that at the morula stage (between 2 and 3 days post coitum, dpc), Sprouty4 is evenly expressed throughout the cells. Similarly, the trophectoderm marker Cdx2[175] and the pluripotency marker Nanog displayed a uniform distribution, as previously described[176]. At the blastocyst stage however (between 3.5 and 4.5 dpc), Sprouty4 was exclusively detected in the interior cells that will form the so-called inner cell mass, the future embryo proper. In a similar manner and as previously reported[176], the expression of the stem cell-associated factor Nanog was restricted to the inner cell mass of the blastocyst. In contrast, the caudal-type homeodomain transcription factor Cdx2 was concentrated in the outer cells of the blastocyst. Consistently, Cdx2 has been reported to be specifically expressed in the trophectoderm of the blastocyst and its expression is maintained within the proliferating extraembryonic ectoderm[175]. Overall, the whole-mount immunofluorescence experiments show that not only is Sprouty4 highly expressed in ES cells, it can also be detected *in vivo* within the inner cell mass of preimplantation embryos.

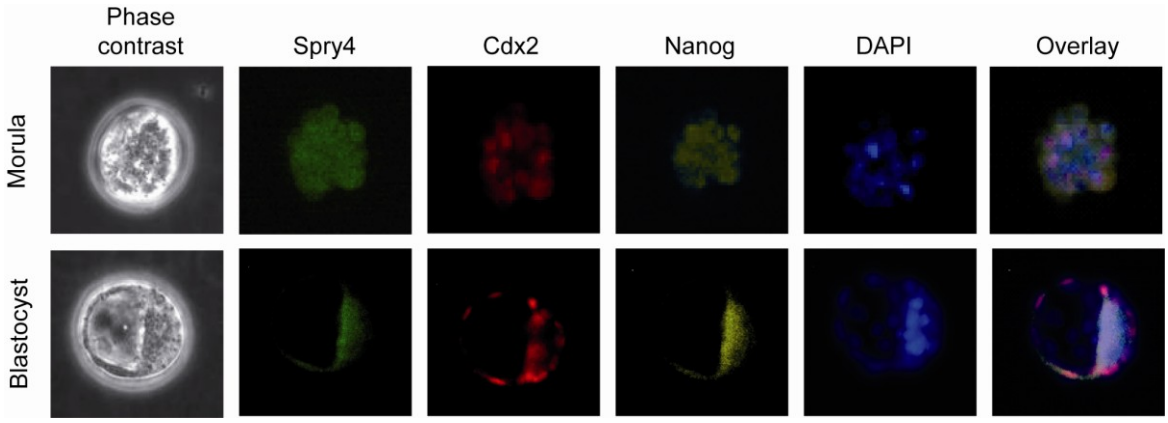


Figure 14: Expression of Sprouty4 in vivo. Immunofluorescence images of preimplantation embryos (before E4.5).

Top panels: late morula. **Bottom panels:** early blastocyst. Embryos stained with primary antibodies against Spry4 (green), Cdx2 (red) and Nanog (yellow). 4, 6-Diamidino-2-phenylindole (DAPI) (blue) was used as a specific nuclear label. All panels are shown at equal magnification.

2.4 The Sprouty4 promoter is regulated by pluripotency-associated factors in ES cells

To understand the molecular basis of *Sprouty4* regulation in ES cells, reporter gene assays were performed. These assays enable the measurement of the regulatory potential of a DNA sequence by linking a promoter sequence to a detectable reporter gene such as that encoding

for the luminescent *Firefly* luciferase (luc). A 1,2kb fragment upstream of both the human (hSpry4(-1200/-1)-luc) and the murine *Sprouty4* (mSpry4(-1200/-1)-luc) genes was found to be able to efficiently drive luciferase expression in mouse ES cells (Figure 15A). A plasmid containing the 2.4kb upstream region of the mouse Oct4 gene fused with the luciferase gene (mOct4-luc) and a construct containing 1kb of the 5'-flanking region and 601bp of the 5'-untranslated region of the murine Klf4 gene (mKlf4-luc) have been reported to induce high expression levels of luciferase[177,178]. Of note, the luciferase expression levels induced by the Sprouty4 promoter are comparable to that of *Oct4* and *Klf4* promoters. *In silico* analyses (Genomatix and TfSearch softwares) of the 1kb region upstream of the transcription start site of *Sprouty4* promoter region enabled the identification of potential binding sites for Klf4, Nanog and Stat3 (Figure 15B) and the vast majority of these predicted binding sites were conserved among human and mouse. The consensus sites of Nanog, Klf4 and Stat3 have been previously described to be 5'-(G/A)(G/A)(G/A)(C/A/G)(C/T)CATT(T/G/A)(C/A)C-3', 5'CCCCACCC-3' and 5'-TTC(C/T)N(A/G)GAA-3' respectively[143,154,179].

Interestingly, most of the conserved putative binding sites for Nanog, Klf4 and Stat3 were predicted to be clustered in the 500bp upstream region of the *Sprouty4* gene. Therefore, a shorter version of the m*Spry4*(-1200/-1)-luc construct was generated and assessed for its capacity to drive luciferase. The m*Spry4*(-500/-1) promoter was able to drive luciferase expression as effectively as the m*Spry4*(-1200/-1) construct, indicating that putative regulators of *Sprouty4* are probably binding within this region as suggested by the *in silico* analyses (Figure 15B). Moreover, all promoter constructs were found to be downregulated upon differentiation with retinoic acid treatment, which correlates well with the decreased expression of endogenous Sprouty4 observed previously at mRNA level under the same conditions (Figure 12A).

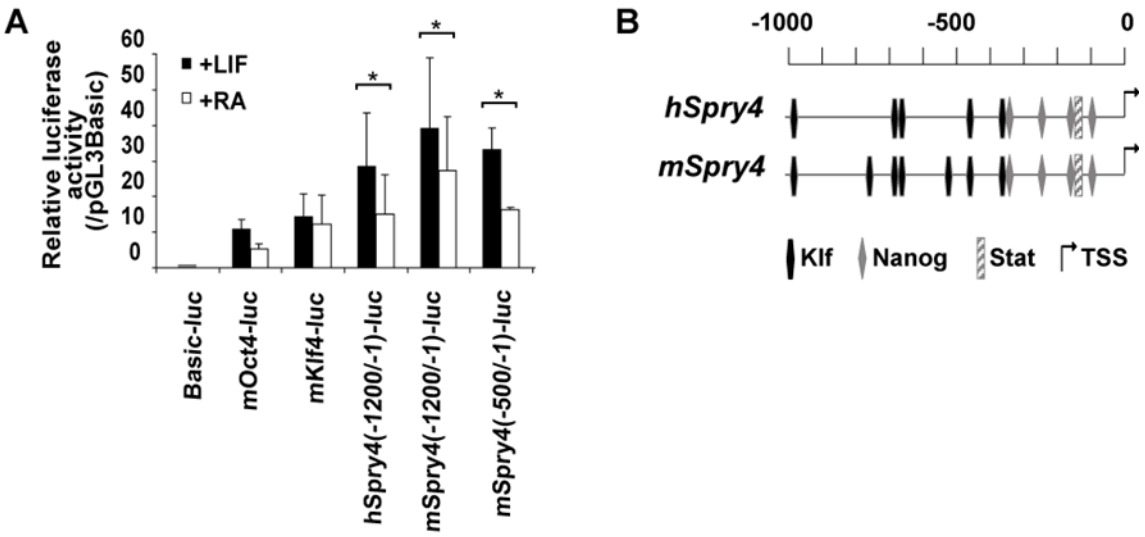


Figure 15: The Sprouty4 promoter is highly active in ES cells.

(A): Schematic representation of reporter constructs used to assay for promoter activity. A 1.2kb- or a 500bp-region upstream of Spry4 transcription start site was inserted upstream of a luciferase gene. The reporters were transiently transfected in the presence of LIF or RA for one day. Luciferase activities are shown relative to those of pGL3-Basic. Bars represent the means ± SD of three independent experiments. Asterisk (*) indicates a p-value ≤0.05. **(B):** Schematic representation of the human and mouse Spry4 promoters with potential binding sites for Klf, Nanog and Stat. Abbreviations: TSS, Transcription start site.

To assess whether or not the pluripotency-associated factors Nanog, Klf4 and Stat3 regulate the Sprouty4 promoter by direct binding, chromatin immunoprecipitation assays (ChIPs) were performed followed by qRT-PCR analyses using ES cells cultured in the presence of LIF or RA for two days. Using antibodies against Nanog, Klf4 and Stat3, it could indeed be demonstrated that all three transcription factors were able to bind directly onto *Sprouty4* promoter (Figure 16). Nanog was enriched by almost 8-fold at the *Sprouty4* promoter, an enrichment comparable to that of *Sall4* (10-fold), a well-known target of Nanog[180,181]. A dramatic enrichment of more than 200-fold was observed for Klf4 at the *Sprouty4* promoter while a 3-fold enrichment was seen for *Zfp42*, a described target of Klf4[182]. Stat3 was as enriched at the *Sprouty4* promoter as it was at its known target *Zfp42*[183]. Notably, Oct4 was found to be only mildly enriched at the Sprouty4 promoter in comparison to the positive control *Zfp42*[184]. No binding was observed for the unrelated negative control *Actin*. Interestingly, upon retinoic acid-induced differentiation, when the levels of Nanog, Klf4 and Stat3 are reduced[51,143,154], Nanog did not bind anymore to *Sprouty4* promoter while the binding of Klf4 and Stat3 was dramatically reduced, highlighting the specificity of these interactions on Sprouty4 promoter. Taken together, these data demonstrate that in undifferentiated ES cells, the *Sprouty4* promoter is bound *in vivo* by the pluripotency markers Nanog, Klf4 and Stat3. This strongly suggests that Sprouty4 may be regulated by these same factors.

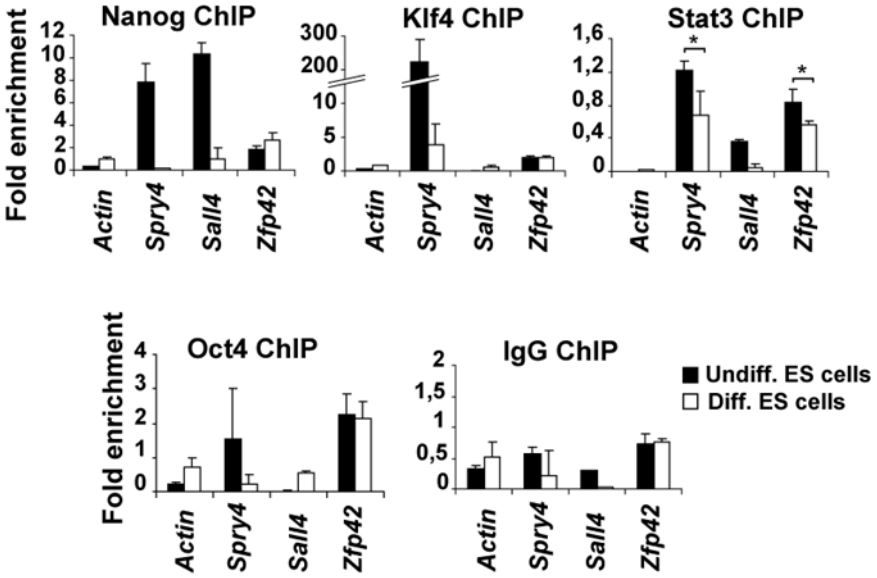


Figure 16: Nanog, Klf4 and Stat3 regulate Sprouty4 expression by direct binding in vivo.
ChIP assays were performed using antibodies against Klf4, Nanog, Oct4, Stat3 and IgG (negative control) with extracts derived from ES cells treated (Diff. ES cells) or not (Undiff. ES cells) with RA for two days. Enrichment is given as fold enrichment over input. Asterisk (*) indicates a p-value ≤0.05.

Nanog has been shown to directly bind to the promoter of Sprouty4 *in vivo* (see above). To assess how Nanog expression affects Sprouty4 levels, mouse ES cells were infected with lentiviral constructs to generate cells that stably express either Nanog or Oct4 as a positive control. ES cells were infected with the empty vector MSCV serving as a negative control. As both expression constructs were tagged, a comparable expression of exogenous Nanog (~40kDa) and Oct4 (~38kDa) by western-blot using an antibody against the HA tag could be confirmed (Figure 17A). Next, the effects of Nanog and Oct4 overexpression on gene expression by qRT-PCR were analyzed. It has been shown that Nanog overexpression could strongly increase the mRNA levels of *Bmp4* but that the expression of other genes such as *Sox2* with associated Nanog binding sites remained unchanged[170]. Accordingly, following Nanog overexpression, a solid upregulation of *Bmp4* expression by two-fold was found here. Moreover, the mRNA levels of other known Nanog targets such as *Esrrb*[185] and *Nr0b1*[186] were also slightly increased (1.2 and 1.3-fold respectively). *Esrrb* has been shown to be able to be upregulated by Oct4 in a comparable manner[170] and showed indeed an increased gene expression of almost 50% following Oct4 overexpression. Strikingly, the overexpression of Nanog led to a dramatic increase of *Sprouty4* mRNA levels of three-fold compared to the control cells. Altogether, the ChIP and overexpression results show that Nanog is able to positively regulate Sprouty4 by direct binding. Interestingly, although Oct4 seemed to bind only weakly

to the Sprouty4 promoter (Figure 16), its overexpression led to a similarly dramatic upregulation of Sprouty4 mRNA than that of Nanog overexpression. It is probable that the effects of Oct4 overexpression on Sprouty4 are indirect, and presumably via upregulation of Nanog as it has been shown that Oct4 can bind to Nanog promoter[187].

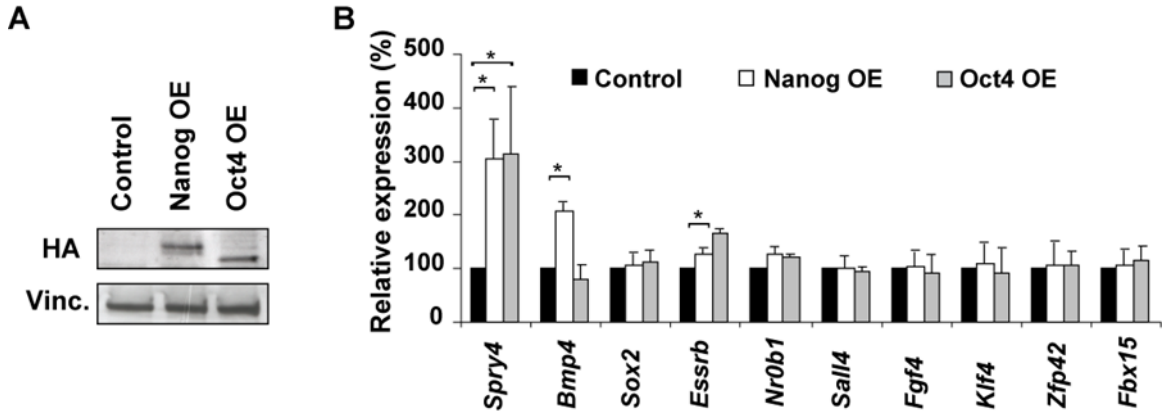


Figure 17: Nanog overexpression upregulates Sprouty4.

(A): Western blots using anti-HA antibody showing the expression of Nanog-HA and Oct4-HA in ES cells after lentivirus infection. Vinculin was used as a loading control. **(B):** Changes in gene expression after Nanog and Oct4 overexpression. Data are expressed as average expression levels ±SD relative to ES cells infected with the empty vector. Asterisk (*) indicates a p-value ≤0.05. Abbreviation: OE, overexpression; Vinc, Vinculin.

2.5 Alteration of the MAPK pathway and ES cell proliferation by Sprouty4 misexpression

The expression of Sprouty4 tightly correlates with the differentiation state of ES cells. Its high expression in undifferentiated ES cells and its acute downregulation upon differentiation suggest that it may play a role in ES cell pluripotency (Figure 12). This hypothesis was investigated by examining the effects induced by the stable expression of a wild-type (WT) version of Sprouty4 or a mutated form of Sprouty4 (Figure 18A). The Sprouty4RBDΔ mutant construct lacks the Raf-binding domain (RBD) in the region comprising amino acids 191-218. It has been previously shown that the deletion of this domain gives rise to a dominant negative construct that is able to impair the capacity of all Sprouty proteins to inhibit the ERK/MAPK pathway upon growth factor stimulation[101]. Mouse ES cells were thus infected with retroviral constructs to generate cells constitutively expressing either Sprouty4 or Sprouty4RBDΔ. Western blot analyses confirmed the expression of both constructs in comparable amounts as revealed by the expression of the C-terminal His tag (Figure 18B). Moreover, ES cells stably expressing exogenous Sprouty4 showed a sustained decrease in activated Phospho-ERK expression confirming that the ERK pathway is significantly inhibited. On the contrary, ES cells

expressing the Sprouty4RBDΔ mutant form showed a higher expression of Phospho-ERK, reflecting the inability of Sprouty4 to inhibit the ERK/MAPK signaling. Accordingly, c-Myc, a downstream target of Phospho-ERK, displayed a similar expression pattern in the two cell lines[188]. Upon exogenous Sprouty4 expression, c-Myc was dramatically reduced to a barely detectable level. The protein levels of the phosphorylated form of c-Myc were consistent with these observations while the protein amounts of total ERK were assessed as a control and found to be similar in all cell lines. Interestingly, the phosphorylated active form of Akt, a downstream target of the PI3K pathway that can be activated through Ras[189], seemed to be slightly downregulated in the Sprouty4 cell line and increased in the Sprouty4RBDΔ cells. Quantification of the western-blot confirmed that Phospho-Akt was decreased by 0.7-fold in Spry4 cells while it was increased by 1.4-fold in the Sprouty4RBDΔ ones. Moreover, the analysis of known PI3K/Akt downstream targets, Hif1a[190] and Nag1[191], revealed that their expression was strongly increased in ES cells stably expressing the dominant negative form of Sprouty4 (by 2.2- and 1.7-fold respectively) (data not shown). In contrast, the phosphatase and tensin homolog gene PTEN, an inhibitor of the PI3K/Akt pathway[192], showed no change in expression in the different cell lines.

The cells expressing exogenous Sprouty4 or its mutated form appeared morphologically normal with no obvious evidence of differentiation during early passages (Figure 21, upper panels). Similarly to normal pluripotent ES cells or cells infected with the empty vector, the different cells lines retained aspect of tight round colonies specific to undifferentiated ES cells. However, during routine culture, the Sprouty4RBDΔ mutant cell line was noted to have a higher tendency to differentiate when confluence was reached but could nevertheless be continuously passaged when plated at low density ($5x10^5$ cells/10cm-plate). This may indicate a possible role for Sprouty4 in ES cell self-renewal. Other groups have similarly reported that the disruption of stem cell-associated genes such as *Esrrb*, *Zfp206, Klf5* or *Pim-1*/*Pim-3* had no immediate apparent morphological effects on ES cells[165,193,194,195]. To test whether misexpression of Sprouty4 had an impact on cell proliferation, a cumulative growth rate curve was performed over nine days in normal ES cell culture conditions (with LIF) including all lines in parallel.

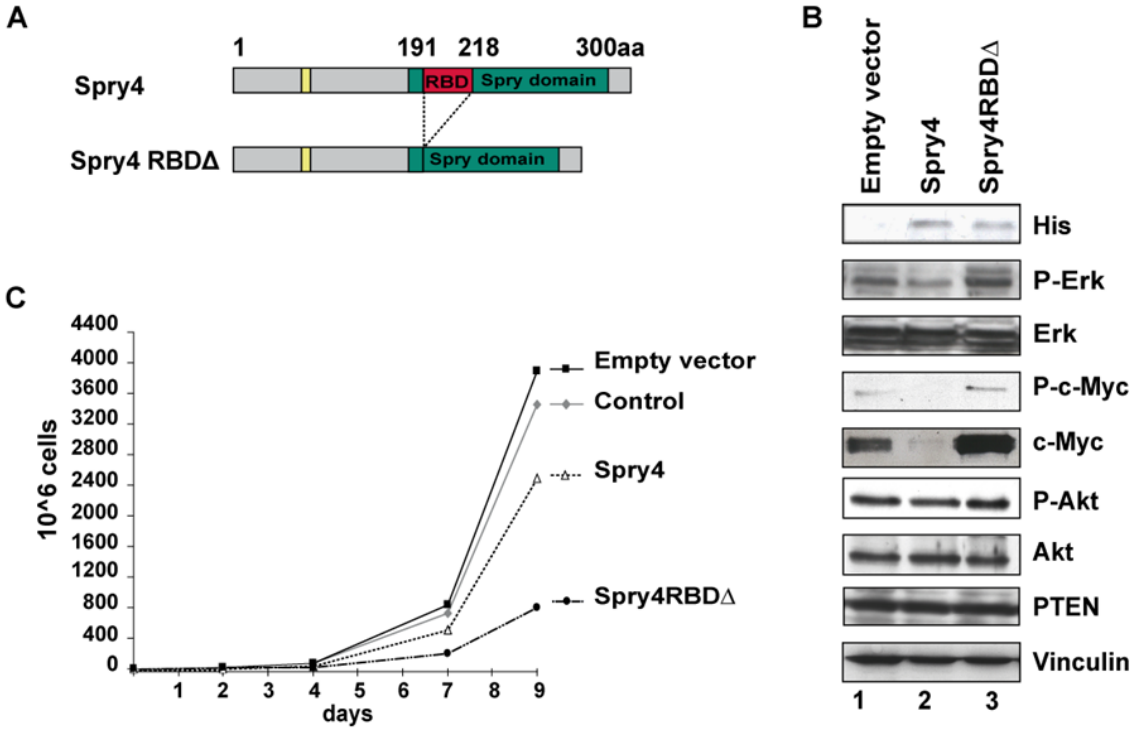


Figure 18: Sprouty4 dominant negative mutant ES cells exhibit altered proliferation rate.
(A): Important domains of the murine Sprouty4 protein. The mutant Spry4RBDΔ lacks the Raf-binding domain (RBD). **(B):** Western blot for the C-terminal His tag showing the sustained expression of the different constructs, for Phospho-Erk, total Erk, Phospho-c-Myc, c-Myc, Phospho-Akt, Akt and PTEN in the stable Spry4 cell lines. **(C):** Growth of uninfected ES cells (control), cells infected with empty vector, Spry4 and Spry4RBDΔ mutant ES cell lines. At each time point, the mean number of cells for duplicate plates is shown.

Compared to the controls (uninfected ES cells or cells infected with the empty vector), ES cells expressing the WT version of Sprouty4 showed only a slight decrease in the growth rate (Figure 18C). The ERK/MAPK pathway has been described to induce both differentiation and proliferation[50]. It is thus possible to hypothesize that a stronger inhibition of this pathway induced by the presence of exogenous Sprouty4 may prevent ES cells from differentiating, but might slightly decrease the proliferation rate at the same time. Importantly, expression of the Sprouty4RBDΔ mutant led to a dramatic reduction of the cellular growth rate. The inability of Sprouty proteins, in the presence of the dominant negative Sprouty4RBDΔ form, to inhibit the ERK/MAPK signaling leads to a higher activation of the pathway, which may thereby promote differentiation. The decreased growth rate may therefore be explained by the fact that the cells are still proliferating but at a much slower rate than normal ES cells as they are already differentiated. Indeed, it has been shown that differentiated cells have a slower growth rate than undifferentiated ones[196]. Notably, no major differences in apoptosis rate were observed with the different cell lines.

2.6 Modified levels of pluripotency-associated genes, altered spontaneous differentiation and self-renewal capacities induced by Sprouty4 misexpression

Next, the ability of Sprouty4 and Sprouty4RBDΔ ES cells to maintain pluripotency was assessed. When injected into nude mice, pluripotent ES cells have been shown to be able to give rise to tumors called teratomas that show evidence of differentiation into cells representing the three germ layers[16]. To test whether the Sprouty4 and Sprouty4RBDΔ ES cell lines are still able to form teratomas, $1x10^6$ cells diluted in Matrigel were subcutaneously injected into mice (in collaboration with Dr K. Eckert, EPO Berlin). Tumors were surgically dissected from the mice 26 days after the injection and the sections were stained with hematoxylin and eosin. For both Sprouty4 and early passage Sprouty4RBDΔ ES cell lines, the derived teratomas exhibited various differentiated tissue types spanning from keratinocytes to cartilage and secretory epithelium, respectively representing the three germ layers (ectoderm, mesoderm and endoderm) (Figure 19). This demonstrates that, at least in part, the ability to maintain pluripotency is unaltered for the Sprouty4 and the early passage Sprouty4RBDΔ mutant ES cells.

To investigate more precisely if pluripotency is maintained at the molecular level in Sprouty4 and Sprouty4RBDΔ ES cells, qRT-PCR analyzes were performed to study the expression levels of several ES cell pluripotency-associated genes in the different cell lines (Figure 20A). ES cells expressing the WT version of Sprouty4 showed an increase of mRNA expression levels of all four pluripotent markers assessed (*Oct4*, *Nanog*, *Zfp42* and *Klf4*). *Oct4* expression was dramatically increased by almost 2.5-fold while the expression of *Nanog*, *Zfp42* and *Klf4* was augmented by 1.5-fold.

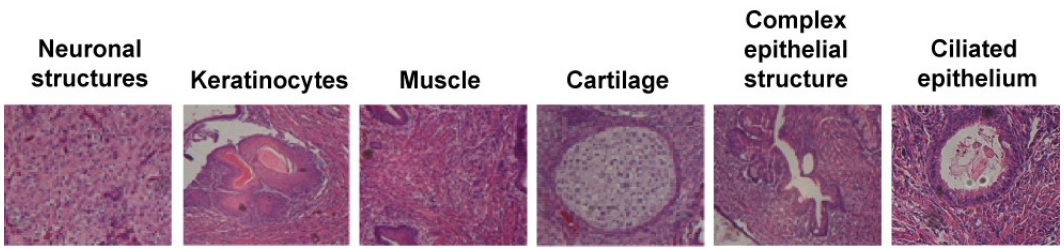


Figure 19: Teratomas derived from Sprouty4 and early passage Sprouty4RBDΔ ES cells.
Examples of tissue types present in teratomas include neuronal structures and keratinocytes (ectoderm-derived), muscle and cartilage (mesoderm-derived) and complex epithelial structure and ciliated epithelium (endoderm-derived).

Conversely, the Sprouty4RBDΔ cell line presented much lower mRNA levels of *Nanog* and *Klf4*, both being significantly decreased by almost 50%. Thus, these data suggest that consti-

tutive expression of Sprouty4 may preferentially direct ES cells towards the pluripotent lineage while the expression of the mutant Sprouty4RBDΔ seems to prevent cells from maintaining a proper pluripotent state. However, the expression changes that are observed are not sufficient to prevent the maintenance of undifferentiated ES cells in the presence of LIF. This observation is consistent with other studies where misexpression of pluripotency-associated genes such as *Oct4*, *Fbx15* and *Zfp206* failed to completely thwart ES cell self-renewal in normal culture conditions with LIF and serum[136], [197], [194].

Interestingly, the expression of *e-Ras* (a Ras-like gene that is specifically expressed in undifferentiated ES cells[198]) was greatly decreased by more than two-fold in the Sprouty4 expressing cells compared to the control cells. It has been shown that e-Ras could promote cell growth by interacting with the PI3K pathway[198]. Accordingly, the PI3K downstream target phospho-Akt has been found to be downregulated in *e-Ras* null ES cells. As a consequence, the downregulation of *e-Ras* in Sprouty4 cells could account for the decrease in phospho-Akt protein levels observed previously and could at least partially account for the lower proliferation rate shown in Figure 18C.

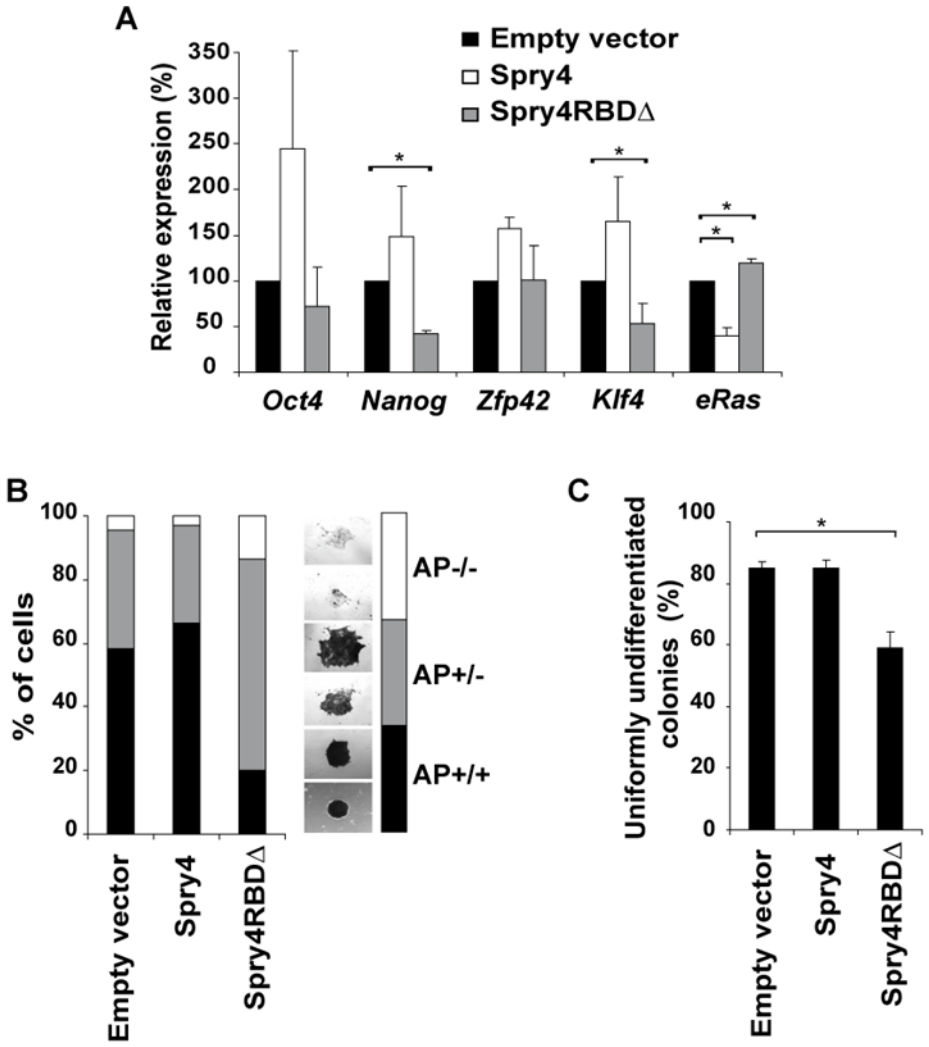


Figure 20: Sprouty4 dominant negative mutant ES cells exhibit altered proliferation and self-renewal capacities.

(A): Expression of pluripotency markers was assessed by qRT-PCR in the Spry4 and Spry4RBDΔ ES cell lines. Data (representative of two experiments) are presented as expression level relative to the ES cells infected with the empty vector. **(B):** Percentage of cells present at day six after plating 1000 cells in standard ES cell culture conditions. Colonies were fixed, stained and scored for levels of alkaline phosphatase expression (AP+/+ high, AP+/- medium or AP-/- low). **(C):** Colony-plating assay of Spry4 and mutant ES cell lines. Cells were plated at low density to generate individual colonies that were scored for differentiation by alkaline phosphatase staining. Data are expressed as the average ±standard deviation of three separate experiments. Asterisk (*) indicates a p-value ≤0.05.

To confirm whether the reduced proliferation rates are primarily caused by either differentiation or growth defects, an alkaline phosphatase (AP) staining and a colony plating assay were performed. The degree of differentiation in the various ES cell lines was assessed by alkaline phosphatase, a well-known stem cell marker, whose expression reflects the undifferentiated state of ES cells[199]. The cells were scored as AP+/+ when expressing a high level of AP (undifferentiated), AP+/- when having a medium level of AP (partly differentiated), or AP-/-

when little AP staining was visible (fully differentiated). As observed in Figure 20B, the Sprouty4 expressing cells showed an increased amount (~10%) of AP positive cells (high and medium AP levels) compared to the control cells infected with the empty vector, which means that the overexpression of Sprouty4 is able to enhance the undifferentiated state of ES cells. Moreover, these results suggest that the slight decrease observed previously in the growth rate is essentially caused by a higher inhibition of the ERK/MAPK pathway by Sprouty4 rather than a consequence of differentiation. In contrast, the Sprouty4RBDΔ cell line exhibited a higher amount of partly/fully differentiated cells (40% more than the control), confirming that the lower growth rate observed previously (Figure 18C) is primarily due to aberrant ES cell differentiation.

To assess the ability of the ES cell lines to self-renew, a colony-plating assay was performed. This experiment scores for clonogenicity, which is the ability of ES cells to seed colonies of fully undifferentiated cells as assessed by alkaline phosphatase staining (see above). All cell lines were plated at clonal density and the number of AP positive colonies (AP+/+) present after four days was ascertained. Similarly to the control, more than 80% of the cells constitutively expressing the WT version of Sprouty4 were able to form uniformly undifferentiated colonies when plated at very low density. Therefore, the self-renewal capacity of the Sprouty4 cell line is not altered, further confirming that the decreased proliferation rate previously observed is not due to sporadic differentiation but rather to altered proliferation. In contrast, corroborating the previously observed inappropriate spontaneous differentiation phenotype, the Sprouty4RBDΔ cell line demonstrated a markedly lower clonogenicity (20% less) than the empty vector control (Figure 20C).

2.7 Sprouty4 misexpression alters the onset of differentiation-related markers and the sensitivity of ES cells towards differentiation

Overexpression of the pluripotency-associated factor Nanog has been shown to sustain ES cell pluripotency in the absence of LIF[143]. Conversely, *Nanog*-deficient ES cells differentiated into extraembryonic endoderm lineage, which indicates that the precise levels of critical regulators can govern the fate of ES cells. To determine whether the abnormal expression of Sprouty4 affects the potential of ES cells to differentiate, the various Sprouty4 cells lines were differentiated by withdrawing LIF and treating with retinoic acid for the days indicated while the morphological changes were observed. It was previously shown that the misexpression of Sprouty4 did not seem to affect the morphology of ES cells in normal LIF culture conditions.

However, after one day of retinoic acid treatment, it was observed that the cells expressing exogenous WT Sprouty4 were still exhibiting an ES cell-like appearance while the control (ES cells infected with the empty vector MSCV) were already starting to differentiate, adopting a more flattened morphology (Figure 21A). After two days of treatment, the Sprouty4 cells were eventually beginning to adopt a differentiated morphology while the controls were already fully differentiated. At day three after treatment though, the ES cells constitutively expressing Sprouty4 were as differentiated as the control cells. These data suggest that the sustained expression of exogenous Sprouty4 is able to delay differentiation induced by retinoic acid in ES cells. Conversely, the Sprouty4RBDΔ mutant cell line already showed flattened cells after only one day of treatment, while the control cells were just starting to exhibit morphological changes. At day two and three, ES cells expressing the Sprouty4RBDΔ mutant seemed to be fully differentiated. Altogether, my data show that the expression of a dominant negative mutated form of Sprouty4 can increase the sensitivity of ES cells to differentiation at least in part by disrupting the repressive activity of Sprouty4 on the ERK/MAPK pathway.

To quantify the observed alteration in differentiation potential of all Sprouty4 cell lines, ES cells were cultured for five days in suspension to induce formation of differentiated embryoid bodies and total RNA was extracted for qRT-PCR analyses. In contrast to the control cells, the Sprouty4 expressing cells presented much lower levels (less than 10%) of lineage markers for endoderm, mesoderm, ectoderm and trophectoderm (Figure 21B). In Sprouty4 ES cells, only the *Fgf5* expression level was comparable to the control. This might be explained by the fact that *Fgf5* is one of the earliest lineage markers to be detected[200]. Thus, overexpression of Sprouy4 seems to strongly delay the expression of most of the lineage markers upon differentiation. In contrast, while the expression of certain embryonic lineage markers such as *Sox17*, *Tbx6* and *Fgf5* was mildly increased in Sprouty4RBDΔ cells (between 10 and 40% more), a significant increase of almost two-fold was observed in the case of the extraembryonic tissue markers *Cdx2* and *Fgfr2*. These results imply that the disruption of the ability of Sprouty proteins to inhibit the ERK/MAPK pathway seems to induce aberrant differentiation of ES cells preferentially into the trophectoderm lineage.

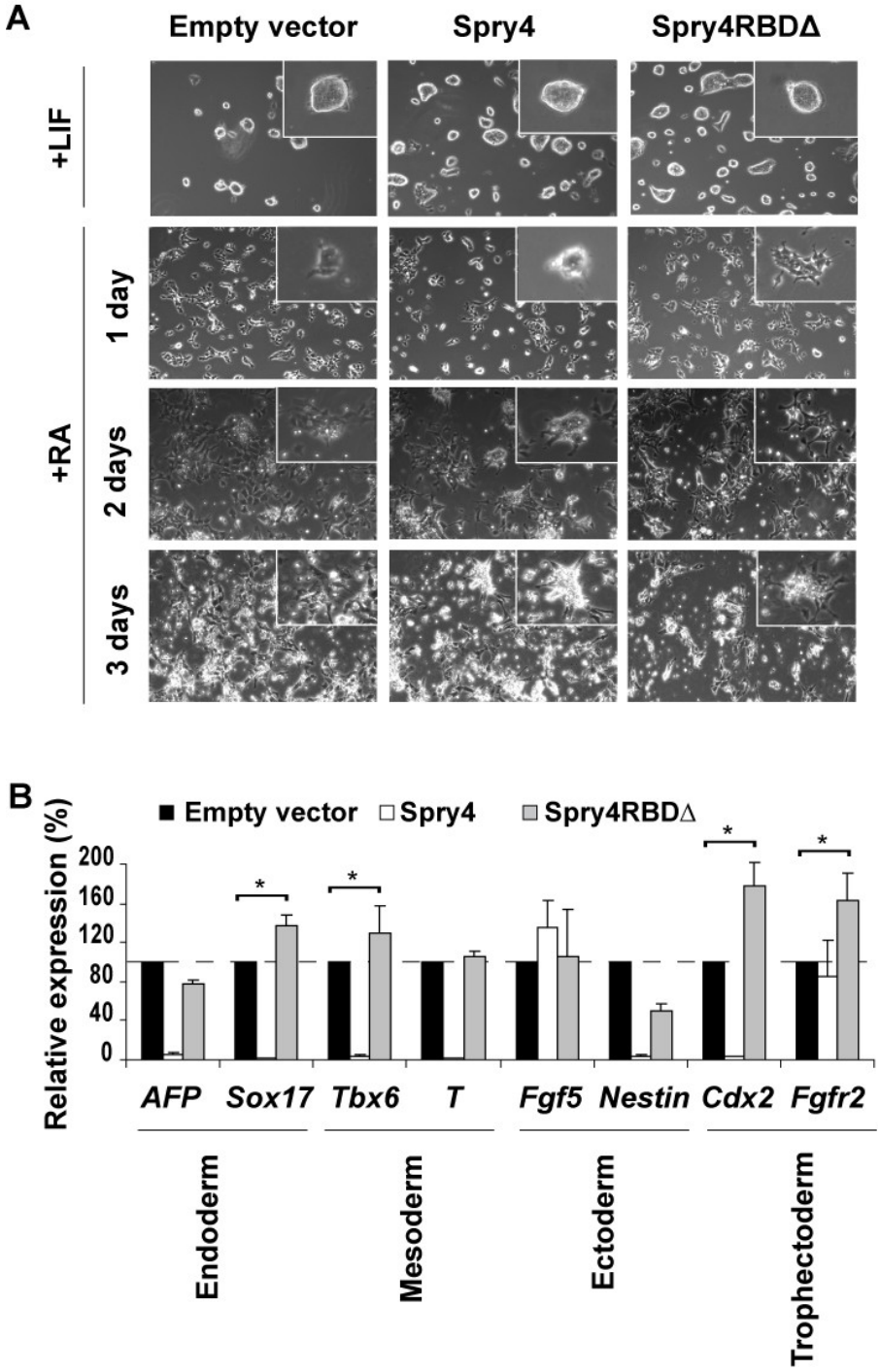


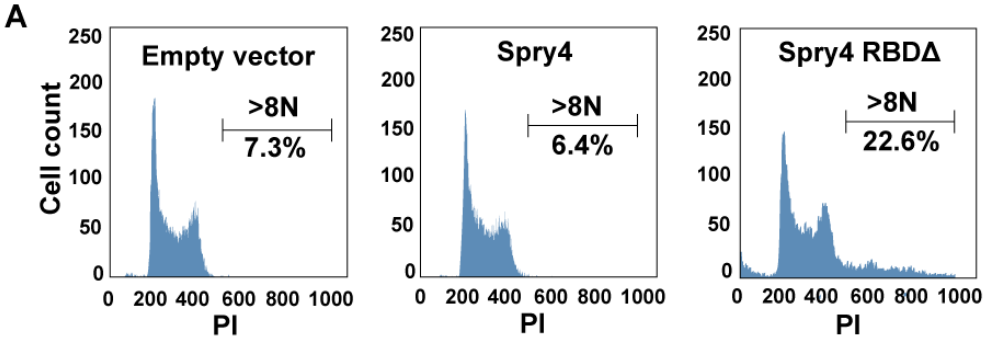
Figure 21: Impact of Sprouty4 misexpression on ES cell differentiation.

(A): Cultures of Spry4 and Spry4RBDΔ cells and empty vector control were treated with retinoic acid for the number of days indicated. Pictures were taken everyday. **(B):** Expression of lineage markers was assessed by qRT-PCR in Spry4 and Spry4RBDΔ mutants cell lines after differentiation into embryoid bodies for five days. Gene expressions were normalized to cells infected with the empty vector and represented as average ±standard deviation of two separate experiments. Asterisk (*) indicates a p-value ≤0.05.

2.8 The dominant negative form of Sprouty4 induces trophectoderm differentiation and progressive polyploidy in ES cells

Following implantation, cells overlying the inner cell mass, called the polar trophectoderm, continue to proliferate and form the extraembryonic ectoderm that contains trophoblast stem (TS) cells and the diploid ectoplacental cone, while the mural cells cease division and form trophoblast giant cells[201]. Further differentiation of the trophoblast lineage generates the labyrinth, spongiotrophoblast and glycogen cells of the mature chorioallantoic placenta[202]. Wild-type ES cells do not normally differentiate to trophectoderm in culture, reflecting the property of their founder population, the inner cell mass[203]. However, the previous experiment has shown that the Sprouty4RBDΔ mutant cells could induce the expression of trophectodermal markers (*Cdx2* and *Fgfr2*) following differentiation into embryoid bodies. To confirm whether the expression of the dominant negative form of Sprouty4 can lead to the formation of extraembryonic tissue, flow cytometric analyses were performed. DNA contents were evaluated by staining with propidium iodide which binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Flow cytometric analyses revealed that the Sprouty4RBDΔ mutant cells displayed a high percentage (22.6%) of polyploid cells (>8N) compared with the control cells which were mostly diploid (2N) or tetraploid (4N) prior to cell division (Figure 22A). Polyploidy has been shown to be characteristic of trophoblastic giant cells which present swollen nuclei and extensive cytoplasmic spreading[204]. These results further confirm that the expression of the Sprouty4RBDΔ mutant leads to the aberrant induction of the trophectoderm lineage. Moreover, this polyploidy seemed to be progressive upon passaging (data not shown) as previously observed for a *Sox2* dominant negative mutant[205]. In contrast, the Sprouty4 cell line did not present any major differences in the DNA contents compared to the control. Similarly, the cell cycle profile of the Sprouty4 expressing cells showed no significant alteration in comparison with the control, both presenting a typical stem cell-like profile where most cells are in the S phase[19] (Figure 22B). Consistent with the observed progressive polyploidy, the Sprouty4RBDΔ mutant cells showed a much lower rate of cells in S phase and more cells in G_2/M. This has also been observed with ES cells that were differentiated into embryoid bodies[19]. Interestingly, a slightly higher rate of apoptosis was detected for the Sprouty4RBDΔ mutant cells in comparison to the control cells (4.1% versus 1.9% in subG_1), suggesting that some cells resorted to apoptosis when unable to self-renew, as previously reported[206].

Concomitantly with the formation of polyploidy, Sprouty4RBDΔ mutant cells expressed significant higher mRNA levels of *p21* and *Cyclin D1*, two cell cycle regulators of the G_1 phase (Figure 22C). Normally, ES cells have much lower expression levels of G_1 phase regulators compared with differentiated cells as ES cells are devoid of G_1 restriction checkpoint. Thus, upregulation of these factors in Sprouty4RBDΔ cells suggests that the disruption of Sprouty4 function leads to the formation of progressive polyploidy by releasing the normal repression of G_1 phase regulators in ES cells.



B

	Empty vector	Spry4	Spry4RBDΔ
$subG_1$	1.9%	1.3%	4.1%
G_1	34.6	34.8	27.8
S	36.6	39.9	24.5
G_2/M	19.6	17.6	20.1

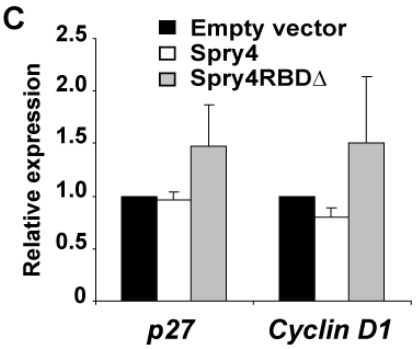


Figure 22: Constitutive expression of Spry4RBDΔ in ES cells leads to trophectoderm differentiation and polyploidy formation.

(A): Cell cycle profiles of ES cells stably expressing the empty vector, Spry4 or Spry4RBDΔ. Stable cell lines were harvested, fixed in 70% ethanol and stained with propidium iodide (PI). DNA contents were analyzed by flow cytometry. Octoploid (8N) and multiploid DNA contents are indicated. **(B):** DNA contents analyzed by flow cytometry. Results represent percentages of cells in each stage of the cell cycle. **(C):** Relative mRNA expression levels of cell cycle regulators assessed by qRT-PCR in ES cells constitutively expressing Spry4 WT or Spry4RBDΔ as compared to cells infected with the empty vector. The expression was normalized against Actin.

Altogether, these results confirm the AP staining and the colony formation assays in that the expression of a dominant negative form of Sprouty4 leads to differentiation of ES cells preferably into extraembryonic lineages. This further demonstrates that the MAPK inhibitor Sprouty4 not only supports ES cell pluripotency but also plays a role in the decision between self-renewal and lineage commitment.

3 Discussion

In this study, novel regulators of ES cell pluripotency have been identified and a role for the MAPK inhibitor Sprouty4 in the self-renewal capacity and differentiation potential of ES cells has been investigated. Sustained expression studies of a wild-type version and a dominant negative form of Sprouty4 have been used to understand how the tight regulation by Sprouty4 of pivotal signaling pathways can govern the fate of ES cells in the decision between self-renewal and lineage commitment.

3.1 Identification of Sprouty4 as a novel regulator of ES cell pluripotency by microarray analysis

Whole genomic transcriptional profiling of pluripotent versus differentiated ES cells enabled the identification of Sprouty4 as a potential novel regulator of murine ES cell fate. Ramalho-Santos *et al*.[207] and Ivanova *et al*.[208] were the first to use microarrays in an attempt to identify "stemness" genes, proposed to be important for conferring the functional characteristics of stem cells. Both groups compared embryonic stem cells, neural stem cells and hematopoietic stem cells with their differentiated counterparts. They speculated that the intersection of genes collectively enriched in all three types of stem cells may represent putative stemness genes. Remarkably, when comparing both studies, only six genes overlapped. More strikingly, a comparison of the two gene lists to another subsequent independent study showed only one transcript commonly identified[209] (α6-integrin). It has been proposed that the extreme discrepancies in the lists of putative stemness genes may be explained by the fact that stemness genes, if such genes exist at all, may be expressed at only low levels in stem cells[209]. Therefore, cross-validation of lists generated independently by investigators that use different cells and analytical methodologies is crucial. Another possibility is that different stem cell types might use different gene networks to achieve pluripotency[50].

Interestingly, 227 transcripts were shared by the list of mouse ES cell-enriched genes reported by Ramalho-Santos *et al*.[207] and the first analysis of human ES cells published by Sato *et al*.[210]. These findings suggest that the regulatory programs underlying ES cell fate seem, at least partially, evolutionarily conserved at a molecular level. Recently, using a newly described computational strategy, a group could identify more than 380 overlapping genes that were previously shown to represent the core expression signature of ES cells in other profiling studies[211,212]. It is thus likely that molecular determinants of pluripotency versus differen-

tiation may involve a specific cluster of factors and signaling regulators working in concert to regulate the fate of stem cells[18].

The rational of my study was that the use of three ES cell differentiation methods, (i.e., LIF removal, embryoid bodies formation, RA addition) would enable the identification of genes that are critical for the early steps of pluripotency loss independently of the lineage specified. *Sprouty4* was found to be quickly repressed by more than 16-fold upon differentiation using all three protocols and this result was further confirmed by qRT-PCR. Interestingly, although known pluripotency-associated factors including *Zfp42*, *Klf4* and *Sox2* were present in my list, it was intriguing not to find other well-described genes such as *Nanog*. This might be explained by the fact that ES cells were differentiated for only three days and therefore the detection of rather early differentiation events was favored. Moreover, in contrast to retinoic acid, the differentiation triggered by LIF removal or embryoid bodies formation is relatively slow. Indeed *Nanog* does decrease after three days of differentiation using all three procedures but to a much lesser extend than other stem cell markers such as *Zfp42*. Besides, a gene might be strongly downregulated in one or two of the differentiation types but not in all three.

3.2 Sprouty4 is highly expressed in pluripotent cells

It has been reported that Sprouty4 could be detected in the mouse embryo as early as E8.5 and later in a number of adult tissues such as heart, brain, lung, kidney and skeletal muscle[73]. *In silico* expression analyses using human expressed sequence tags (ESTs) previously suggested that human *Sprouty4* mRNA was expressed in ES cells and in certain types of colon cancer, head and neck tumor, melanoma and pancreatic cancer[213]. In my PhD thesis, I used western-blot experiments to demonstrate that Sprouty4 is indeed highly expressed in undifferentiated ES cells. Besides, immunofluorescence assays showed that Sprouty4 colocalized with cells positively stained for the pluripotency marker Oct4, which further suggests that Sprouty4 plays a role in the undifferentiated state of ES cells. In the future, it would be interesting to assess whether or not Sprouty4 is expressed in other cell types such as adult stem cells like observed for Oct4[214], which could extend the role of Sprouty4 to other primitive cellular compartments and eventually cancer stem cells.

In endothelial cells, human Sprouty1 and Sprouty2 have been shown to be widely distributed throughout the cell but predominantly found in perinuclear regions, vesicular structures and in the plasma membrane[88]. In response to stimulation with EGF, mouse Sprouty4 translocated from the cytoplasm to membrane ruffles (i.e., a disarrangement of the surface plasma membrane caused by reorganized actin cytoskeleton) in COS1 cells[85]. In great contrast, I was

able to demonstrate that Sprouty4 is present in both cytoplasmic and nuclear compartments of undifferentiated ES cells as revealed by immunofluorescence assays. These observations were further confirmed in western-blot experiments following ES cell subcellular fractionation where Sprouty4 was found in the same cellular compartments as the nuclear marker Lamin A and the cytoplasmic marker Tubulin. These findings raise the question as to whether the nuclear localization of Sprouty4 is specific to ES cells and which functional consequences this particular localization may have for Sprouty4 in controlling pluripotency.

Whole-mount immunofluorescence experiments showed that Sprouty4 was evenly expressed in all blastomeres of embryos at the morula stage (2-3 dpc) but later exclusively detected in the inner cell mass of blastocysts (3.5-4.5 dpc). The same expression pattern has been observed for the pluripotency-associated factors Nanog and Sall4[176,180]. In contrast, although Klf5 has been shown to be essential for blastocyst development and the normal self-renewal of ES cells, immunohistochemical analyses have shown that it is ubiquitously expressed in both morula and blastocyst[195]. It has been proposed that Klf5 functions upstream of *Nanog* and *Cdx2* and that its role in the trophectoderm layer is essential for the implantation process of blastocysts[195]. This could account, at least in part, for its localization in both the inner cell mass and the trophectoderm of preimplantation embryos.

Interestingly, confocal microscopy studies of the MAPK signalling intermediate Erk2 revealed a polarized expression at the apical membrane of peripherally localized blastomeres at the 8-cell stage[176]. At later stages, Erk2 was evenly distributed throughout all cells of the morula and blastocyst, similarly to Sprouty4, Nanog and Cdx2. This asymmetric Erk2 expression in 8-cell embryos, when blastomeres have not yet adopted a clear fate, suggests that MAPK signalling may act as an upstream effector of trophectoderm fate[176]. Because I have found that the disruption of Sprouty activity in ES cells leads to the formation of trophectoderm, it would be interesting to assess the expression of Sprouty4 in embryos prior to the morula stage. Moreover, as Sprouty4 signals upstream of Erk2, its differential expression in the blastocyst could suggest a much more complex role than the inhibition of the MAPK pathway alone.

3.3 Regulation of Sprouty4 by pluripotency-associated factors

Using *in silico* analyses, potential putative binding sites for Nanog, Klf4 and Stat3 were identified on the *Sprouty4* promoter in the 500bp region upstream of the transcription start site. ChIP assays established that Nanog, Klf4 and Stat3 were indeed able to bind *in vivo* onto the *Sprouy4* promoter. No direct binding could be found for Oct4 in my experimental conditions

but a weak binding cannot be totally excluded as the fold enrichment found for *Sprouty4* was only slightly weaker than that of the well-defined Oct4 target *Zfp42*. Interestingly, genome-scale location analyses of human ES cells have previously suggested that *Sprouty1* was targeted by Nanog, but not by Oct4 and Sox2, whereas another member of the Sprouty family, *Spred1*, was bound by Nanog, Oct4 and Sox2[215]. Of note, ChIP on Chip experiments relying on streptavidin affinity capture of tagged transcription factors have suggested that *Sprouty4* promoter could be bound by Nanog, Klf4, Sox2, Nr0b1 and Nac1, but not by Oct4, Zfp42 , Zfp281 and c-Myc in mouse ES cells[215]. Intriguingly, *Sprouty2* was found to be potentially bound by the same factors than *Sprouty4* in addition to Oct4, Zfp281 and Zfp42[215]. The results obtained in the present study are however the first proof that *Sprouty4* is indeed bound *in vivo* by several pluripotency-associated factors including Nanog, Klf4, Stat3, and probably others.

Lentiviral expression of exogenous Nanog increased the expression level of *Sprouty4* in ES cells by three-fold, suggesting that the direct binding of Nanog onto *Sprouty4* observed in the ChIP assays is capable of activating *Sprouty4*. Although it has been suggested that Nanog overexpression did not influence the basal levels of activation of Erk[142], it was speculated here that Nanog overexpression would increase Sprouty4 levels, which in turn would lead to Erk repression. It possible that the effects on Erk are only seen in a restricted time window after overexpression or the altered phospho-Erk levels may only be visible upon growth factor stimulation. Whether Nanog expression can influence Erk would need to be addressed in the future. Besides, the increase in *Sprouty4* expression following Oct4 overexpression was rather surprising. Either Oct4 can upregulate *Sprouty4* directly, which could not be confirmed here by ChIP; or the effects induced by Oct4 ovexexpression are indirect. As it has been shown that Oct4 could bind to the *Nanog* promoter[187], one hypothesis may be that Oct4 overexpression increases the expression levels of *Nanog*, which in turn activates *Sprouty4*. This will have to be further investigated.

3.4 Effects of Sprouty4 expression on the growth rate of ES cells

In somatic cell, Sprouty can typically inhibit proliferation[62], however in ES cells Sprouty4 expression resulted in a slightly lower growth rate than the control with no alteration of the apoptosis rate. This is a probable consequence of the observed decreased levels of activated phospho-Akt as it has been shown that the Akt pathway mediates proliferation[216]. It is however intriguing that Sprouty4 cells can proliferate almost normally despite marked low c-Myc levels considering that Myc proteins are known to control cell growth[217]. Similarly,

findings have reported that Sprouty2 overexpression led to a reduction in growth factor-mediated cell proliferation[95].

In contrast, sustained expression of the dominant negative Sprouty4 mutant was found here to dramatically decrease cell growth. Similar reduced proliferation rates were observed in ES cells following the triple depletion of *Klf2*, *Klf4* and *Klf5,* three members of the stem cell-associated Krüppel-like factor family[154]. This indicates that, as seen here for Sprouty4, the disruption of factors that are important for ES cell pluripotency is typically accompanied by proliferation defects.

3.5 Sprouty4 constitutive expression protects ES cells from differentiation

Constitutive expression of the wild-type version of Sprouty4 induced higher expression levels of the pluripotency-associated markers *Oct4*, *Nanog*, *Zfp42* and *Klf4*. Consistently, Sprouty4 ES cells were less prone to spontaneous differentiation as assessed by alkaline phosphatase staining. Similarly, overexpression of the serine/threonine kinases Pim1 and Pim3 in ES cells, two signaling regulators that have been shown to modulate ES cell pluripotency, increased the rate of undifferentiated colonies compared to control cells[165]. It was speculated here that Sprouty4 may protect ES cells from spontaneous differentiation by sustaining the expression of pluripotency factors while preventing the onset of lineage-specific markers. Indeed, it has been shown that Oct4 can hinder trophectoderm formation by inhibiting the expression of the trophectoderm marker *Cdx2* in the inner cell mass of embryo and likewise in ES cells[218]. Although the constitutive expression of Sprouty4 in the ES cells did not significantly alter the expression levels of lineage-specific markers in LIF culture condition (data not shown), one cannot rule out that higher levels of Sprouty4 might induce differentiation towards certain lineages as it has been shown that a two-fold overexpression of Oct4 was needed to induce primitive endoderm and mesoderm formation[136].

Besides, the sustained expression of Sprouty4 was able to delay the morphological changes normally occurring upon retinoic acid differentiation. Consistently, the forced presence of Nanog was found to delay, rather than block, the differentiation of ES cells[142]. It is possible that high levels of overexpression are required to completely prevent differentiation. The delayed morphological alterations seen in Sprouty4 cells upon differentiation are likely explained by the dramatic low expression levels of all lineage-specific factors assessed after embryoid bodies formation. So far, the ERK pathway has only been linked to trophectoderm differentiation and primitive endoderm specification[121,176]. The fact that Sprouty4 sustained expression prevents the proper formation of ectoderm, endoderm and mesoderm tissues

suggests a more complex role for Sprouty4 in ES cells than just inhibition of the ERK pathway. Even though the embryoid bodies looked morphologically normal, only *Fgf5* and *Fgfr2* were found to be expressed after five days of differentiation. Interestingly, both are growth factor-related genes. Likewise, ES cells overexpressing the pluripotency factor *Esrrb* showed no induction of the mesoderm marker *Brachyury* and only a weak increase of *Fgf5* upon LIF withdrawal as compared to control cells[193].

There is always a concern with sustained expression that similarly to transient overexpression, the high-level of expression leads to non-physiological effects. However in the Sprouty4 cell line, no dramatic apparent differences in morphology or growth rate were detected. In addition, the outcomes of the constitutive wild-type Sprouty4 expression are consistent with the inverse results that were observed in the dominant negative mutant studies.

3.6 A dominant-negative form of Sprouty4 predisposes ES cells to differentiation

Although *Sprouty4* and *Sprouty2* KO mice are viable[116,219], the early lethality of *Sprouty4*/*Sprouty2* double KO mice precludes a thorough investigation of the stem cell compartment. Silencing of human Sprouty2 in adrenal cortex adenocarcinoma SW13 cells did not alter the amounts of endogenous Sprouty1 and Sprouty4[98]. In contrast, while overexpression of Sprouy4 has been shown to block branching morphogenesis of the lung, no abnormalities were observed in *Sprouty4*-deficient lung, suggesting that loss of Sprouty4 may be compensated in specific cells by other members of the Sprouty family[116]. The expression of either a dominant negative version or a WT version of Sprouty4 in ES cells did not however alter the expression of neither Sprouty2 nor Sprouty1, whereas Sprouty3 was not detected (data not shown). This suggests that at least in pluripotent ES cells, Sprouty4 expression does not affect other Sprouty family members, however it does not rule out that Sprouty2 or Sprouty1 may have supportive roles.

Expression of a dominant negative form of Sprouty4 did not dramatically increase the expression levels of lineage-specific factors in normal culture conditions except for the trophectoderm marker *Cdx2* where a mild increase of 1.5-fold was observed (data not shown). The almost normal expression of lineage-associated markers is consistent with the apparent morphology of the cells which formed round-shaped colonies with clear delimited contours specific to ES cells. Besides, Sprouty4RBDΔ mutant cells were still able to induce teratomas when injected subcutaneously in mice. Whether late passage Sprouty4RBDΔ cells that have progressively accumulated polyploid cells would still be able to generate teratomas has to be

determined. Moreover, as I have previously demonstrated that the Sprouty4RBDΔ cells could delay differentiation but not completely prevent it, it is probable that differences between teratomas generated from the control and the mutant would be revealed at an earlier time point than 26 days. It was speculated that the formation of teratomas might be slower in the mutant cells than in the control and/or that the variety of the differentiated tissues found in the tumors might vary as well. This will have to be further investigated. Similarly to Sprouty4 RBDΔ, early passage *Klf5* KO ES cells could generate teratomas which exhibited successful differentiation into the three germ layers[195]. Likewise, *Klf5* KO ES cells showed only slight decrease in expression levels of differentiation markers such as *Fgf5*, *Brachyury* or *Cdx2* and no change at all for other markers including *Gata4*[195].

More importantly, expression of the Sprouty4RBDΔ mutant led to decreased expression levels of at least the pluripotency-related factors *Nanog* and *Klf4* whereas spontaneous differentiation in the presence of LIF was dramatically augmented. Hamazaki *et al.*[146] have shown that the ERK pathway could mediate *Nanog* repression upon ES cell differentiation into primitive endoderm. In contradiction, others have shown that ES cells cultured in the presence of the MEK inhibitor PD98059 presented unaltered steady-state *Nanog* mRNA levels indicating that the ERK signaling does not repress *Nanog* expression[142]. The fact that *Nanog* expression was significantly increased following Sprouty4 constitutive expression and decreased in the presence of the dominant negative form of Sprouty4 strongly pleads for an inhibitory activity of the ERK signaling upon *Nanog*. Sprouty4 constitutive expression markedly represses the ERK pathway thus probably leading to the release of the inhibition of *Nanog* expression by the pathway. In contrast, the presence of the Sprouty4 mutant prevents the inhibition of the ERK signaling by all Sprouty proteins, which leads to a hyper-stimulated state of the pathway and a stronger inhibition of *Nanog* expression.

The self-renewal capacity of Sprouty4 ES cells was markedly decreased as assessed in the clonal assay. These findings suggest that Sprouty4RBDΔ ES cells are more prone to spontaneous differentiation and have a decreased self-renewal capacity probably as a consequence of decreased pluripotency- and self-renewal-related factors such as *Nanog* and *Klf4*. Consistently, ES cells expressing the Sprouty4RBDΔ mutant version were much more sensitive to retinoic acid-induced differentiation and adopted the morphology of a fully-differentiated cell only after one day of treatment whereas control cells remained ES cell-like.

At the molecular levels, following embryoid bodies differentiation, the mutant cells showed higher levels of *Sox17* and *Tbx6,* two markers of endodermal and mesodermal tissues respectively. More strikingly, a dramatic increase in the extraembryonic trophectoderm markers

Cdx2 and *Fgfr2* was observed in the Sprouty4RBDΔ cells compared to control cells. It is known that differentiation into embryoid bodies leads to the formation of three-dimensional structures composed of endoderm-, ectoderm- and mesoderm-like cells closely resembling early embryos[168]. Embryoid body formation does not normally lead to high levels of trophectodermal tissue development[168]. However, expression of a dominant negative form of Sprouty4 in ES cells induced significantly elevated expression levels of trophectoderm markers compared with the embryoid bodies derived from control cells. Thus, the expression of mutated Sprouty4 not only prones ES cells to spontaneous differentiation, it also triggers the differentiation of ES cells preferentially into the trophectoderm lineage. This suggests that the level of Sprouty4 expression might ultimately influence the fate of pluripotent ES cells as illustrated in the model proposed in Figure 23.

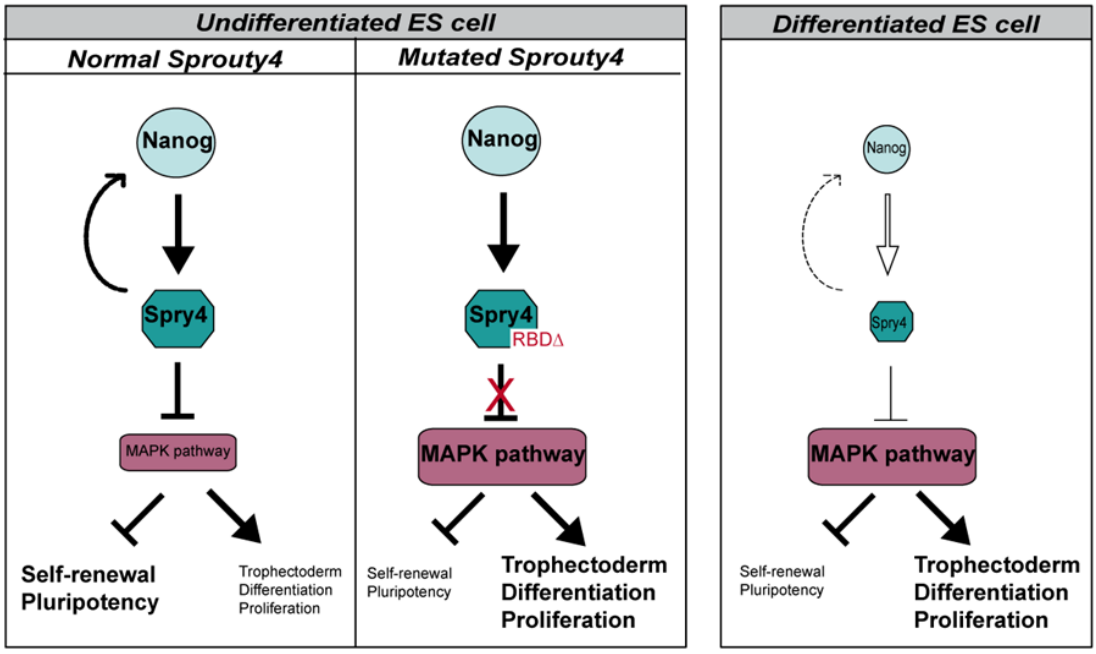


Figure 23: Proposed model for the role of Sprouty4 in ES cell self-renewal and differentiation. ***Left and middle panels:***
In undifferentiated ES cells, high levels of Nanog, Klf4, Stat3 and probably other pluripotency-associated factors upregulate Sprouty4 expression leading to a strong inhibition of both the MAPK pathway and trophectoderm differentiation, while self-renewal and pluripotency are favored. Impairment of Sprouty4 activity by the expression of Sprouty4RBDΔ dominant negative mutant releases the repression exercised on the MAPK pathway and induces differentiation into the trophectoderm. ***Right panel:*** Upon differentiation, the downregulation of stem cell factors leads to low levels of Sprouty4 expression. Subsequently, the MAPK pathway is derepressed thus triggering ES cell loss of self-renewal capacity and progressive differentiation.

3.7 Sprouty4 and the cell cycle of ES cells

Flow cytometry analyses have revealed that expression of a dominant negative version of Sprouty4 induced the generation of cells assuming a pattern of gradual differentiation toward trophoblast-like cells reflected by the gradual shift from 2N/4N to 8N/nN in ploidy. These cells might thus serve as a model to investigate the uncoupling of chromosomal duplication and cell/nuclear division seen for trophoblasts. In parallel to polyploidy, increased expression levels of the cell cycle regulators *p27* and *Cyclin D1* were observed in Sprouty4RBDΔ cells. ES cells have a unique cell cycle property with a long S phase and a short G_1 gap phase. Consistent with this unique cell cycle attribute, ES cells have much lower expression levels of G_1 phase regulators (cyclin D1 and D2) and cyclin-dependent kinase inhibitors (p21 and p27) compared with differentiated cells and thus show no classical G_1 restriction checkpoint. Therefore, upregulation of these factors in the Sprouty4RBDΔ ES cell line suggests that the disruption of Sprouty4 function leads to the formation of progressive polyploidy by releasing the repression of these factors in ES cells. This argues that Sprouty4 regulates pivotal signaling pathways that are normally required to repress G_1 regulators in ES cells.

Comparable results were obtained using a Sox2 dominant negative mutant[205], which suggests that, similarly to Sox2, Sprouty4 may help to prevent trophectoderm differentiation and polyploidy formation in ES cells. Intriguingly, progressive G_2/M accumulation and a low level of polyploidy was observed following Sprouty2 overexpressing in a human leiomyosarcoma cell line. It was speculated that Sprouty2 may function as a cell cycle regulator by preventing cells from reentering the G_1 phase of the next cell cycle[95]. This contrasts with the results obtained with Sprouty4 sustained expression in ES cells, which hints towards distinct roles for Sprouty proteins regarding cell cycle regulation. Whereas Sprouty2 may regulate general cell cycle progression, Sprouty4 might help to preserve the atypical cell cycle structure of ES cells with truncated G_1 phase and no G_1 checkpoint. However, the effects of Sprouty2 and Sprouty4 overexpression in ES cells would have to be investigated and compared in the future in order to assess their respective roles in the cell cycle of stem cells.

3.8 Sprouty4 and tumorigenesis

ES cells display many features characteristic of cancer cells including unlimited proliferative capacity, clonal propagation, and a lack of both contact inhibition and anchorage dependence[18]. Recently, it was shown that the growth of certain tumor types depends primarily

on the presence of an undifferentiated stem cell population, called cancer stem cells, that is able to proliferate extensively and form new tumors[220]. Interestingly, some of the cell surface markers expressed by certain cancer stem cells are common to those expressed by normal stem cells, suggesting that cancer may arise in some cases from the malignant transformation of adult stem cells[220,221]. Accordingly, the pluripotency marker Oct4 has been shown to be expressed not only in certain adult stem cells[214] but also in various human tumors[222,223], while promoting tumor growth in a dose dependent manner[224]. It was found that at least one of the four classical pluripotency factors Oct4, Sox2, Klf4 and c-Myc was significantly overexpressed in almost half of the cancer types investigated and that within a given tumor category, these genes were associated with tumor progression or bad prognosis[225]. Recently, a group has proposed a link between genes associated with ES cell identity and the histopathological traits of tumors, suggesting that pluripotency-associated genes may contribute to the stem cell-like phenotypes of tumors such as breast cancer, glioblastoma and bladder carcinoma[211]. It can be thus hypothesized that specific pluripotency regulators may have a positive role in tumorigenesis, possibly contributing to the stem cell-like phenotypes of tumors.

As I have shown that Sprouty4 has a crucial role in the decision between ES cell self-renewal and lineage commitment, it is tempting to speculate that Sprouty4 might exert critical roles in tumorigenesis. Two plausible scenarios are discussed below.

First, it has been reported that Sprouty4 is downregulated in almost one half of human prostate cancers[226]. The decreased Sprouty4 levels may lead to increased expression of c-Myc, in a similar manner to the Sprouty4RBDΔ ES cells, which may then favor cancer cell proliferation as it was shown that high levels of c-Myc are typically associated with rapid proliferation of tumor cells[227]. Furthermore, it has been shown that Sprouty4 deficiency could potentiate Ras-independent angiogenic signals and tumor growth in mouse models, suggesting that Sprouty4 may be considered as a putative tumor suppressor[228]. Therefore, Sprouty4 would be downregulated in tumors addicted to high c-Myc levels, dependent on peripheral blood vessels and showing a low prevalence of a cancer stem cell compartment.

A second scenario is based on my results, which strongly suggest that Sprouty4 is a positive stem cell factor. This is also in concordance with previous ESTs analyses where high levels of Sprouty4 expression were found in stem cells but also in certain types of colon cancer, head and neck tumor, melanoma and pancreatic cancer[213]. In this second hypothesis, primitive cells or cancer stem cells would depend on Sprouty4 action to restrain MAPK-mediated signaling, and therefore c-Myc, in order to attenuate differentiation and favor self-renewal. Con-

sistently, *in vivo* experiments have shown that the balance between self-renewing progenitor cells and committed cells is dependent on Sprouty4 for the formation of ameloblasts in incisors[115] and myoblasts in skeletal muscle[229]. Therefore, Sprouty4 would be upregulated in tumors that display a high dependence on their cancer stem cell compartment and possibly low c-Myc levels. This hypothesis would be true if the most primitive tumors or the cancer stem cells would present higher levels of Sprouty4.

Since it is known that the action of the four mammalian Sprouty proteins is highly context-dependent, it is probable that both scenarios are correct, depending on the tumors. It would hence be of high interest to investigate whether the stem cell compartment of certain tumor models depends on Sprouty4 function.

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

Chemicals were purchased from Merck (Darmstadt), Roth (Karlsruhe) and Sigma (Deisenhofen), enzymes from New England Biolabs (Frankfurt), cell culture media from GIBCO (Karlsruhe) and cell culture plates from TPP (Trasadingen, CH).

4.1.2 Antibodies

Table 2: List of antibodies utilized

Antibodies	Catalogue-Ref.	Company	Concentration	Use
Rabbit αHis(6x)	ab1187	Abcam	1:1000	Western blot
Mouse αc-Myc	9402	Cell signaling	1:1000	Western blot
Rabbit αPhospho-c-Myc	9401	Cell signaling	1:1000	Western blot
Rabbit αErk	9102	Cell signaling	1:1000	Western blot
Mouse αPhospho-Erk (Thr202/Tyr204)	9106	Cell signaling	1:1000	Western blot
Rabbit αAkt	9272	Cell signaling	1:1000	Western blot
Rabbit αPhospho-Akt (Ser473)	9271	Cell signaling	1:1000	Western blot
Mouse αPTEN	6H2	Ziebold/Lees labs	1:3	Western blot
Mouse αHA	H9658	Sigma	1:2500	Western blot
Mouse αVinculin	V9131	Sigma	1:1000	Western blot
Goat αSpry4	sc-18607	Santa Cruz	1:500	Western blot
Rabbit αNanog	RCAB0002PF	CosmoBio	1:300; 4µg;1:50	Western blot; ChIP; IF
Goat αKlf4	sc-12538	Santa Cruz	4µg	ChIP
Rabbit αStat3 (C-20)	sc-482	Santa Cruz	4µg	ChIP
Rabbit-IgG	I5006	Sigma	1:2-1:4	ChIP
Mouse αCdx2	MSK046	Biogenex	1:100	IF
Goat αOct4 (C-20)	sc-8629	Santa Cruz	1:50; 4µg	IF; ChIP
Rabbit αSpry4	NB100-2032	Novus Biologicals	1:50	IF
Goat αMouse Ig-HRP	P0447	DAKO	1:1000	Western blot
Goat αRabbit Ig-HRP	P0448	DAKO	1:2000	Western blot
Rabbit αGoat Ig-HRP	P0449	DAKO	1:2000	Western blot
Sheep αMouse Ig-CY3	515-165-006	Jackson	1:200	IF
Donkey αRabbit Ig-Alexa 647	A31573	Molecular Probes	1:200	IF
Donkey αGoat Ig-Alexa 488	A11055	Molecular Probes	1:200	IF

4.1.3 Oligonucleotides

All oligonucleotides were purchased from Biotez (Berlin) or Metabion (Martinsried).

Table 3: Sequences of oligonucleotides used

Gene	5'-Primer	3'-Primer	Use
mSpry4	GGTACCACCATGGAGCCCCCGGTTC	CCGCGGGAAAGGCTTGTCAG	PCR
mSpry4 Prom	GGTACCACCGGTTTGGGTTGCT	GCTAGCGAGCCGGCAAAGCTCC	PCR
hSpry4 Prom	AAGCTTACCATGGTCAGCCCCCTCCCC	CCGCGGGAAAGGCTTGTCGGGCC	PCR
mKlf4 Prom	CTTTTCCCCTCCCGGTA	TCTCTTGGCCGGGGAA	PCR
Actin	TCTACGAGGGCTATGCTCTCC	GGATGCCACAGGATTCCATAC	qRT-PCR
Klf4	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT	qRT-PCR
Oct4	GGAGAGGTGAAACCGTCCCTAGG	AGAGGAGGTTCCCTCTGAGTTGC	qRT-PCR
Zfp42	GACGGATACCTAGAGTGCATC	GAAGGGAACTCGCTTCCAGAA	qRT-PCR
Brachyury	CCAACCTATGCGGACAATTCATCTGC	GTGTAATGTGCAGGGGAGCCTCGAA	qRT-PCR
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGCT	qRT-PCR
Fgf5	TGTGTCTCAGGGGATTGTAGG	AGCTGTTTTCTTGGAATCTCTCC	qRT-PCR
Fgf4	GGGCATCGGATTCCACCTG	GCTGCTCATAGCCACGAAGAA	qRT-PCR
Gata4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT	qRT-PCR
Spry1	AGTGTGGCAAGTGCAAATGTGGAG	ATGCCCTTGACCAAACACATGCAG	qRT-PCR
Spry2	ACTGCTCCAATGACGATGAGGACA	CCTGGCACAATTTAAGGCAACCCT	qRT-PCR
Spry3	TGCTGCAGGGTGAAGTAAGGAGAA	TATGTGGCTGTGTGCAACCCAATG	qRT-PCR
Spry4	CGACCAGAGGCTCCTAGATCA	CAGCGGCTTACAGTGAACCA	qRT-PCR
AFP	CTTCCCTCATCCTCCTGCTAC	ACAAACTGGGTAAAGGTGATGG	qRT-PCR
Myst4	AGAAGAAAAGGGGTCGTAAACG	GTGGGAATGCTTTCCTCAGAA	qRT-PCR
Tdh	CTGCACAGAGCAAAGCCTCTA	TCAGGAGGTTAGCAAGTCCTAC	qRT-PCR
Trim2	TGGACAGTTCAAAAGTCGTTTCG	AATGCTAACCCACTTGTTGTCAT	qRT-PCR
Arid5b	GTGATGAGTTCGCGCCAAATC	GCTGATAACTTTACCGTCACAGT	qRT-PCR
Nr0b1	TCCTACTGTACCCAGAGTGTG	AGAGGCAACGCTGATCTGC	qRT-PCR
Abca1	AAAACCGCAGACATCCTTCAG	CATACCGAAACTCGTTCACCC	qRT-PCR
Lrrc34	GGCTTGTTGCACTTTCCCAGTCAA	TCTGGCTTTAGTCGGCCACTCTTT	qRT-PCR
Fbx15	CGTGGACGAAATGCCATCGG	ATGATAAAAGCGTCTGCTCACA	qRT-PCR
Sall4	AGCTATTTACCCAAAGGCAAAGT	CGAATCTGTTCCGTAAGCTGG	qRT-PCR
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT	qRT-PCR
Bmp4	AAGGCGCCTGTGTCTTTCAG	GGGCTGTAATCTCCTCATTCTGGTAG	qRT-PCR
Essrb	TTTCTGGAACCCATGGAGAG	AGCCAGCACCTCCTTCTACA	qRT-PCR
Fgfr2	GCCTCTCGAACAGTATTCTCC	ACAGGGTTCATAAGGCATGGG	qRT-PCR
Cdx2	CAAGGACGTGAGCATGTATCC	GTAACCACCGTAGTCCGGGTA	qRT-PCR
Eomes	CCTGGTGGTGTTTTGTTGTG	TTTAATAGCACCGGGCACTC	qRT-PCR
Hand1	AGCAAGCGGAAAAGGGAGT	GTGCGCCCTTTAATCCTCTT	qRT-PCR
Tpbp	AAGTTAGGCAACGAGCGAAA	AGTGCAGGATCCCACTTGTC	qRT-PCR
Mash2	TGAATGCAAGCTTGATGGAC	TGGAAGCCCAAGTTTACCAG	qRT-PCR
Ehox	GAGCATCAAAACACCAACTACCT	GCCCACCTTCTCCACTTAATTCT	qRT-PCR
Actin	GCTTCTTTGCAGC TCCTTCGTTG	TTTGCACATGCCGGAGCCGTTGT	ChIP
Spry4	TACAACCCACGCAGCTA	CGGCAAAGCTCCCTGA	ChIP
Sall4	CGAGGAGCGCTAAATTACTAAAA-ACCTGGGAAT	GCAAATGAAATCCGGGCAATAATTA-GCAGAT	ChIP
Zfp42	GAGGTACTGAGATGTGACTGAGTCTCA	CTCCTTGGACCCCTCCCTTTTTAGATG	ChIP
T7-(dT)24	GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT24)		Microarray

4.1.4 Plasmids

Table 4: List of plasmids used

Name	Description
Basic-*luc*	pGL3-Basic vector with minimal promoter used for luciferase gene reporter assay from Invitrogen
mSpry4(-500/-1)-*luc*	vector containing murine Sprouty4 promoter (-500/-1) used for luciferase gene reporter assay
hSpry4-*luc*	vector containing human Sprouty4 promoter (-1200/-1) used for luciferase gene reporter assay
mOct4-*luc*	vector containing murine Oct4 promoter (-2400/-1) used for luciferase gene reporter assay, gift from Dr M. Saito, Kyoto
mKlf4-*luc*	vector containing murine Klf4 promoter (-1000/+600) used for luciferase gene reporter assay
pMSCV	retroviral empty vector with puromycin resistance from Clontech
pMSCV-Spry4	retroviral vector with puromycin resistance expressing WT Sprouty4 (902bp)
pMSCV-Spry4RBDΔ	retroviral vector with puromycin resistance expressing Sprouty4 with a deletion of amino acids 191-218 in the RBD domain
pLenti6/PGK/neo	lentiviral empty vector with RFP tag from System Biosciences
pLenti6/Nanog-HA	lentiviral vector with RFP and HA tags containing full-length human Nanog cDNA from System Biosciences
pLenti6/NOct4-HA	lentiviral vector with RFP and HA tags containing full-length human Oct4 cDNA from System Biosciences
pLP1	packaging vector coding for *gag* and *pol* from Invitrogen
pLP2	packaging vector coding for *env* from Invitrogen
pCMV/VSV-G	packaging vector expressing envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter from Invitrogen

4.2 Methods

4.2.1 Molecular biological methods

Standard procedures for molecular cloning were carried out according to the laboratory manuals from Sambrock et al.[230] and Ausubel et al.[231] and won't be described further. These methods include DNA extraction and purification, DNA digestion by restriction enzymes, dephosphorylation of linearized plasmid, DNA ligation as well as transformation into chemically competent bacteria. The following kits were utilized: DNA plasmid preparation and Gel extraction of DNA plasmid (Genomed).

4.2.1.1 Samples preparation for microarrays

RNAs from undifferentiated or differentiated mouse ES cells were isolated using the RNeasy kit (Qiagen, Hilden) according to the manufacturer's intructions. For the cDNA synthesis, 10µg of each RNA were mixed to 100pmol of the T7-$(dT)_{24}$ primer and denaturated at 70°C for 10min. After being cooled down on ice, the RNA/primer solution was mixed to 1x first strand buffer (Invitrogen, Karlsruhe), 10mM DTT and 2mM dNTP-mix and then incubated at

42°C for 2min. Finally, synthesis was performed using 400U of Superscript II reverse transcriptase (Invitrogen) at 42°C for 1h. The first strand reaction mix was shortly centrifugated and then placed on ice. For the second strand synthesis, 1x second strand reaction buffer, 800µM dNTP mix, 10U *E.coli* DNA ligase (M0205S, NEB), 40U *E.coli* DNA polymerase I (M0209S, NEB) and 2U *E.Coli* RNase H (M0297S, NEB) were added to the first strand reaction and filled up with dH_2O to 150uL. After 2h incubation at 16°C, 10U of T4 DNA polymerase (M0203L, NEB) were supplemented and further incubated for 5min. To stop the reaction, 10µl of 0.5M EDTA were added. The double stranded cDNA was purified by phenol/chloroform extraction and precipitated with ammonium acetate and 100% ethanol.

The cDNA was transcribed *in vitro* into cRNA and biotin-labeled using the BioArray high yield RNA transcription labeling kit (Enzo, Lörrach) according to the manufacturer's intructions except that only half of the amounts were used. To clean up the non-incorporated nucleotides, the cRNA was purified using RNeasy columns (Qiagen). Then 15µg of the cRNA was fragmented through incubation at 94°C for 35min. The hybridization step was performed at the Microarray facility from the Max Delbrück Center (Mrs Born) using the Mouse 430A 2.0 array (Affymetrix).

Analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools development team. The genes were filtered to have at least a 2-fold change in either direction from gene's median value and a *p*-value of the log-ratio variation greater than 0.01. Genes differentially expressed in all 3 types of differentiated ES cells compared to undifferentiated ES cells were identified using the significance analysis of microarrays (SAM) software. SAM uses permutations to estimate the false discovery rate and an adjustable threshold allows for control of the false discovery rate. The lists of genes were analyzed and gene expression was verified by quantitative real-time PCR.

4.2.1.2 RNA isolation, cDNA synthesis and quantitative PCR (qRT-PCR)

The RNeasy kit from Qiagen was used to isolate RNA from cells according to the manufacturer's intructions. The RNA concentration and purity were then determined by a UV-spectrophotometer. To translate the RNA into cDNA, a reverse transcriptase (Superscript II, Invitrogen) was used with 5µg of RNA following the manufacturer's protocol. For the qRT-PCR, 0.5µL cDNA, 0.5µL of 10µM primers mix and 10µL 2x SYBR-Green (ABgene, Hamburg) were analyzed in duplicate on a 96 well-plate. The primers were designed as to have an annealing temperature of 60°C and an amplicon length of 200-300bp in the following pro-

gram (95°C 15min; 94°C 20sec, 60°C 45sec, 72°C 45sec; 40 cycles). The qRT-PCR analyses were carried out with three different cDNAs. See Table 3 for primer sequences.

4.2.1.3 Luciferase assay

Reporter gene assays were performed in order to study the effect of different transcription factors on promoters' activity.

This activity was assessed by the expression of a *Firefly*-luciferase reporter. The different reporter constructs as well as a *Renilla*-luciferase control vector were transfected into ES cells using Lipofectamin2000 (Invitrogen). The cells were harvested the day after transfection in PBS (140mM NaCl, 2.6mM KCl; 4.5mM Na_2HPO_4; 1.4mM KH_2PO_4) and lysed 20min on ice (50mM HEPES pH 7.4, 250mM KCL, 0.1% NP-40, 10% Glycerin, 1mM DTT, 2mM PMSF). Then 10µL of each lysate were transferred in duplicate to a microtiter plate and injected with 50µL of a *Firefly*-luciferase injection solution (25mM Glycyl-glycin, 15mM K_xPO_4, 4mM EGTA, 15mM $MgSO_4$, 1mM DTT, 1mM ATP, 100µM Coenzym A, 75mM Luciferin) and 50µL of a *Renilla*-luciferase injection solution (1.1M NaCl; 2.2mM EDTA; 220mM K_xPO_4; 0.44mg/ml BSA; 0.008% NaN_3; 1.43µM Coelenterazin). The luminescence reaction was measured with a luminometer (Berthold technologies, Bad Wildbad).

4.2.1.4 Chromatin immunoprecipitation (ChIP)

To assess the *in vivo* binding of different transcription factors to the endogenous *Sprouty* promoter, chromatin immunoprecipitations were performed.

In order to have 2-3x10^6 cells per IP at the day of harvest, ES cells were cultivated on MEF-treated 10cm-plates for 48h in the presence or absence of 0.1µM retinoic acid. The cells were then fixed with 270µL Formaldehyde for 10min at room temperature and the reaction was stopped with 1.25mL of 1M Glycin. The cells were resuspended in lysis buffer (5mM PIPES pH 8.0, 85mM KCl, 1% SDS, 0.5mM PMSF, Proteinase inhibitor cocktail (1:1000 from Sigma)) and incubated on ice for 10min. The nuclei were pelleted and incubated on ice for 10min in a nuclei lysis buffer (50mM Tris-HCl pH 8,1, 10mM EDTA, 1% SDS, 0.5mM PMSF, Proteinase inhibitor cocktail) before being cleared by centrifugation. The DNA was fragmented by sonification (Sonifier B12 from Branson Sonic Power) and diluted 1/10 in a dilution buffer (0.01% SDS; 1.1% TritonX; 1.2mM EDTA; 16.7mM Tris-HCl pH 8.2; 167mM NaCl, 0.5mM PMSF, Proteinase inhibitor cocktail).

The chromatin was precleared two times with blocked Protein-A-sepharose (GE Healthcare; 17-0780-01), divided equally among samples and then incubated with the corresponding antibody overnight. The day after, the immunoprecipitates were incubated with blocked Protein A-sepharose for 2h at 4°C on a rotating wheel. The Protein A-sepharose-bound immunoprecipitates were then washed once with a low salt buffer (0.1% SDS, 1% TritonX, 2mM EDTA; 10mM Tris-HCl pH 8.0; 150mM NaCl), once with a high salt buffer (0.1% SDS; 1% TritonX; 2mM EDTA; 10mM Tris-HCl pH 8.0; 500mM NaCl) and finally 5 times with a LiCl wash buffer (0,25M LiCl, 0.5% NP-40; 0.5% Sodium deoxycholate; 1mM EDTA; 10mM Tris-HCl pH 8.0).

After the last wash, 250µL of elution buffer (50mM Tris-HCl pH 8.0; 1% SDS; 10mM EDTA) and 10µl 5M NaCl were added to the Protein A-sepharose and incubated at 65°C overnight shaking to reverse the cross-linking. The protein-DNA complex was then digested by 1µL RNase A (10mg/mL) at 37°C for 1h with light shaking and finally by 2µL Proteinase K (10mg/mL) at 55°C for 2h.

A phenol-chloroform extraction followed by an ethanol precipitation was performed to separate the immunoprecipitated DNA from proteins. A quantitative real-time PCR was finally performed in order to amplify the desired promoters.

4.2.1.5 Site-directed mutagenesis

The deletion of amino acids 191-218 in the Raf-binding domain of Sprouty4 to realize the Spry4RBDΔ retroviral vector was performed using the QuickChangeII site-directed mutagenesis kit (Stratagene). According to the manufacturer's protocol, the pMSCV-Spry4 retroviral construct containing the WT Spry4 full-length cDNA (902bp) was used as template with primers containing the specific desired mutations. After the mutagenesis, the methylated parental DNA template was digested by the restriction enzyme DpnI while the mutated unmethylated DNA was transformed into chemically competent XL-1 cells. The mutations were then verified by DNA sequencing.

4.2.2 Biochemical methods

4.2.2.1 Protein extraction

80-90% confluent cells were washed two times with PBS and lysed in 4 volumes RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP40, 0.5% Natrium desoxycholate, 0.1% SDS) pro-

vided with fresh protein inhibitors (0.5mM PMSF, 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin, 0.4mM Natrium vanadate, 0.4mM NaF) for 20min on ice. The lysed cells were then centrifuged for 15min at 12000g at 4°C and the supernatant was either used directly or stored at -20°C.

4.2.2.2 Protein concentration determination

The protein concentrations were determined by the DC-method from Biorad (München). A standard curve was performed each time using four concentrations of BSA between 0.2 and 1.5mg/mL. In a microtiter plate, 5µL of the standard or protein samples (diluted 1:5) were mixed to 25µL of the Biorad reagent A' and 200µL of the Biorad reagent B. After an incubation of 15min, the absorption at 750nm was measured.

4.2.2.3 SDS-PAGE und Western blot analysis

Equal quantities of proteins were separated using SDS-PAGE (10% Separating gel: 10% acrylamide, 0.32% N,N´-Methylbisacrylamide; 375mM Tris-HCl pH 8.8; 0.1% SDS; 0.1% APS; 0.1% TEMED; Stacking gel: 4% Acrylamid; 0.1% N,N´-Methylbisacrylamide; 125 mM Tris-HCl pH 6.8; 0.1% SDS; 0.1% APS; 0.1% TEMED) in an electrophoresis buffer (25mM Tris-HCl; 20mM Glycin; 2% SDS). The protein samples were diluted 1:1 in 2x SDS-sample buffer (125mM Tris-HCl pH 6.8; 4% SDS; 20% Glycerol; 0.01% Bromophenol blue; 10% β-Mercaptoethanol) and heated at 95°C for 5 min along with the protein markers (Prestained Protein Marker, NEB). The electrophoresis was performed at 100V for 2-3h.

Proteins were then transferred from the gel onto PVDF membranes (Immobilion-P, 45µm, Millipore, Schwalbach am Taunus) by the wet-transfer method overnight (4°C; 30V). The membrane was activated for 15sec in 100% methanol before being stacked with the gel and two layers of Whatman paper avoiding air bubbles and then placed in a transfer chamber with transfer buffer (200mM Glycin; 25mM Tris-HCl; 20% Methanol). Afterwards, the transfer efficiency was assessed by Ponceau staining (2% Ponceau, 1% acetic acid in distilled water) for 1min at room temperature. The membrane was then destained in 1% acetic acid before being washed in 1x TBS.

Non-specific binding sites on the membrane were blocked for 1h at RT in blocking solution (4% skimmed milk powder, 0.1% Tween20 in TBS) with constant shaking. The primary antibodies were then diluted in blocking solution and incubated with the membrane for 2h at RT or overnight at 4°C with constant shaking. After washing three times for 5 min in TBS-T

(25mM Tris-HCl pH 7.4; 137mM NaCl; 5mM KCl; 0.7mM $CaCl_2$; 0.5mM $MgCl_{2;}$ 0.1% Tween20), the membrane was incubated with the horseradish peroxidase-conjugated secondary antibodies diluted in blocking solution for another 45min.

After washing three times in TBS-T, the immuno-reactive bands were visualized using the chemiluminescent detecting ECL reagent (Millipore WBKLS05000). The detection solution (1:1) was applied to the membrane to cover it evenly. After 1min incubation, excess solution was drained from the membrane and the membrane was placed on a flat sheet of saran wrap. The membrane was then exposed to an X-ray film (Hyperfilm, GE Healthcare) for various times and finally developed.

4.2.2.4 Flow cytometry analysis

In order to establish the cell cycle profiles of the different ES cell lines, Propidium iodide (PI) staining was used. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Subsequent flow cytometry analysis was used to evaluate cell DNA content.

For flow cytometry analysis, ES cells were harvested by trypsinization and pelleted by centrifugation at 1200g for 5min at 4°C. The cells were then washed with 10mL ice-cold PBS and pelleted at 1200g for 5min at 4°C. To fix the cells, 1mL of ice-cold 70% ethanol was added dropwise to the cells while slowly vortexing. The fixed cells were incubated at 4°C overnight and then pelleted at 1000g for 10min at 4°C. The pellet was washed with 5mL ice-cold PBS and centrifugated at 1200g for 5min at 4°C. ES cells were resuspended in 500µL Sodium citrate (38mM) and 25µL RNase A (10mg/mL) and incubated at 37°C for 30min. Afterwards, 15µL of PI (1mg/mL) was added and the cells were analyzed by flow cytometry (FACS Calibur) using the FACS Scan software (Becton Dickinson, Heidelberg).

4.2.3 Cell culture methods

4.2.3.1 ES cell culture

ES cells were maintained on feeder-treated tissue culture dishes. Feeder-treated plates were prepared by culturing primary mouse embryonic fibroblasts (MEF) on gelatine-coated dishes. Confluent MEF were treated with mitomycin C (10µg/mL) and then trypsinized. Plates were then washed two times with PBS and stored at 4°C until use.

The R1 ES cell line (a gift from C. Birchmeier) was used in all experiments. ES cells were cultured at 37°C, 7.5% CO_2 at 95% relative humidity in ES medium (DMEM supplemented with 4.5g/L glucose, 15% inactivated batch-tested FCS, 0.1mM 2-mercaptoethanol, 1% Penicillin/Streptomycin, 0.1mM non-essential amino acids and LIF (~1000U/mL)). All products were from Gibco (Karlsruhe) except LIF (COS cells stably expressing LIF, a gift from C. Birchmeier). ES cells were plated at a density of 1-2x10^6 cells per 10cm-plates and splited every 2-3 days. The medium was changed everyday.

4.2.3.2 ES cell differentiation

For spontaneous differentiation, ES cells were plated on gelatin-coated plates at a density of 2x10^6 cells per 10cm-plate in ES cell differentiation medium (IMDM, 10% inactivated batch-tested FCS, 1% Penicillin/Streptomycin, 0.1mM non-essential amino acids and 300µM monothioglycerol (Sigma, Taufkirchen)). The medium was changed everyday.
For retinoic acid differentiation, ES cells were cultured as for spontaneous differentiation but additionally treated with retinoic acid (0.1µM) for the days indicated. The medium was changed everyday.
Embryoid bodies were formed as in the so-called mass culture technique by seeding a single-cell suspension of ES cells at 60000cells/mL in a low adherence cell culture dish in ES cell differentiation medium. The medium was changed every two days.

4.2.3.3 ES cells transfection with Lipofectamin

ES cells were transfected using Lipofectamin2000 from Invitrogen. Cells were plated on gelatin-coated 12 well-plates at a density of 200 000 cells/well. The day after, 4µL Lipofectamin2000 were diluted in 96µL OptiMEM and incubated for 5min at RT. In the meantime, 1.2µg DNA were diluted in OptiMEM to obtain a final volume of 100µL. The Lipofectamin-OptiMEM mix was combined to the DNA-OptiMEM mix and incubated for 20min at RT. The final mix was then distributed dropwise on the cells. The medium was changed 24h after transfection with or without the addition of 0.1µM retinoic acid for another day.

4.2.3.4 Retrovirus production using Phoenix cells

The Phoenix packaging cell line was used to produce ecotropic viruses. This cell line stably expresses genes for the viral Gag, Pol and Env proteins. Murine retroviruses are produced via the transfection of adapted plasmids and can infect cells possessing ecotropic receptors. RNA

flanked by plasmid encoded ψ-sequences, are packed in viruses and retrotranscribed in DNA following infection of target cells. Finally, the DNA is stably integrated in the genome of the cells.

Phoenix cells were transfected with the $CaPO_4$ method. Cells were plated the day before transfection at $5x10^6$ cells/10cm-plate in standard medium (DMEM supplemented with 1g/L glucose, 10% inactivated FCS, 1% Penicillin/Streptomycin and 0.1mM non-essential amino acids). The medium was changed 2h before transfection. For a 10cm-plate, 30µg DNA were mixed to 62µL 2M $CaCl_2$ and 438µL dH_2O. The DNA/$CaCl_2$/dH_2O mix was distributed dropwise to 500µl 2xHBS (280mM NaCl, 1.5mM NaHPO4, 50mM HEPES; pH 7.13) while vortexing. After 30min incubation at room temperature, the solution containing the precipitated DNA was pipetted dropwise onto the cells. The day after, the medium was changed. In the evening, Phoenix cells were given 6mL of fresh medium and incubated overnight at 32°C. The day after, the virus containing-supernatant was harvested and either directly used for infection or stored at -80°C.

4.2.3.5 ES cell infection with ecotropic viruses

ES cells were plated at a density of 10^6cells per 10 cm-plate on feeder-treated dishes one day before infection. The day after, the 6mL of virus-containing phoenix supernatant were diluted 1:1 in ES medium, sterile filtered (0.45µm) and provided with polybrene (final concentration 10µg/mL). ES cells were then infected using the phoenix supernatant. In the afternoon of the same day and on the morning of the following day, the same procedure was repeated to achieve a triple infection in order to increase the infection efficiency. In the afternoon after the triple infection, fresh ES cell medium was given to the cells. To select the infected cells, puromycin was added to the medium two days following the last infection at a final concentration of 2µg/ml.

4.2.3.6 Lentivirus production using 293TN cells

The 293TN producer cell line which has been transformed with the SV40 large T antigen was utilized in order to produce high titers of pseudoviral particles. Producer cells are transiently co-transfected with the expression (pLenti6/PGK/neo) and packaging vectors (pLP1, pLP2 and pCMV/VSV-G). Expression constructs packaged in pseudoviral particles are secreted by producer cells in the culture medium and are used directly to transduce expression constructs into target cells. Following transduction into the target cells, the expression construct is re-

verse transcribed and integrated into the genome of the target cell. The plasmid pLP1 enables the Rev-dependent expression of g*ag* and *pol* to form the structure and for the replication and integration of the lentivirus respectively while pLP2 encodes the Rev protein. Rev interacts with the RRE (Rev response element) present on pLP1 to induce *gag* and *pol* expression, and on the pLenti6/PGK/neo expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles. The pCMV/VSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter, thus replacing lentiviral *env* gene. Viral particles, VSV-G protein pseudotyped, mediate viral entry through lipid binding and plasma membrane fusion.

The 293TN producer cells were transfected with the $CaPO_4$ method. Cells were plated the day before transfection at $4x10^6$ cells/10cm-plate in DMEM supplemented with 4.5g/L glucose, 10% inactivated FCS, 1% Penicillin/Streptomycin and 0.1mM non-essential amino acids. For a 10cm-plate, 12µg of the expression vector, 6µg of pLP1, 3µg of pLP2, 3µg of pCMV/VSV-G were mixed to 62.5µL 2M $CaCl_2$ and 413.5µL dH_2O. The DNA/$CaCl_2$/dH_2O mix was distributed dropwise to 500µl 2xHBS (280mM NaCl, 1.5mM NaHPO4, 50mM HEPES; pH 7.13) while vortexing. The mix was then incubated for 2min at 37°C to facilitate the formation of crystals. After the incubation, the solution containing the DNA was pipetted dropwise onto the cells. Chloroquine was then added to the plates at a final concentration of 25µM in order to optimize the transfection by inhibiting lysosomal DNases. After 16h, the plates were washed once with fresh medium and 293TN cells were given 6mL of fresh medium. The day after, the virus containing-supernatant was harvested and either directly used for infection or stored at -80°C.

4.2.3.7 ES cell infection with lentiviruses

ES cells were plated at a density of $5x10^5$cells per 10 cm-plate on feeder-treated dishes one day before infection. The day after, the 6mL of virus-containing phoenix supernatant were diluted 1:1 in ES medium, sterile filtered (0.45µm) and provided with polybrene (final concentration 10µg/mL). ES cells were then infected using the supernatant. On the morning of the following day, the same procedure was repeated to achieve a double infection, which increases the infection efficiency. On the third day, ES cells were harvested and analyzed by western-blot and real-time PCR.

4.2.3.8 Cumulative growth curve

To quantify proliferation, ES cells were plated in duplicate at a density of 10^6cells per 10cm-plate on feeder-treated dishes in ES cell medium. At day 2, 4, 7 and 9, ES cells were dissociated with trypsin and triterated to a single cell suspension, counted and replated at 10^6cells per 10 cm-plate. The medium was changed everyday.

4.2.3.9 Alkaline phosphatase (AP) staining

To assess pluripotency, ES cells were plated on MEF-treated 24 well-plates at a density of 5000 cells/well in ES cell medium. At day six, the cells were washed with ice-cold PBS and then fixed in 4% paraformaldehyde for 15min at room temperature. After three washes with PBS, 400µL of X-Phos/NBT detection buffer (100mM Tris-HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$) were added and the cells were incubated for 15min at RT. After removal of the detection buffer, 400µL of X-Phos/NBT reaction solution (Boehringer, Dortmund) were pipetted onto the cells and the plate was incubated for another 15 min at RT protected from light. After three washes with PBS, AP staining was quantified and photos were taken. Cells were scored as displaying either a high/homogenic AP-staining (AP+/+), or a medium/heterogenic AP-staining (AP+/-), or a weak/absent AP-staining (AP-/-).

4.2.3.10 Colony forming assay

To assess self-renewal, ES cells were plated at clonal density (150 cells/well) on MEF-treated 6 well-plates in ES cell medium. At day six, the cells were assessed for AP-staining (see above). Colony formation efficiency was calculated by counting the number of AP-positive clones exhibiting high and homogenic staining (AP+/+).

4.2.3.11 Statistical analysis

Student's t test was used to determine statistical significance (MS Office Excel). P-values less than 0.05 were considered significant.

4.2.4 Immunocytochemical methods

4.2.4.1 Immunofluorescent staining of ES cells

ES cells were plated at a density of 60000cells/well in 2-well chamber slide (LabTek, Stadtlohn) and cultured in ES medium (+LIF) for 3 days. The chambers were previously coated with poly-L-Lysine (50µg/mL in PBS) for 1h at room temperature. The cells were then washed twice with PBS and fixed for 15min with 4% paraformaldehyde. Afterwards, the ES cells were again washed twice with 1mL PBS and then the residual aldehyde groups were inactivated with 1mL of 15mM Glycin for 5min. The cells were rinsed with 1mL PBS and then permeabilized with 1mL 0.2% Triton X-100 for 10min at room temperature and washed once with PBS. The cells were then incubated for 20min in 1mL of blocking solution (0.2% gelatin in PBS). A dilution of the first antibody in blocking buffer containing 16.5% FCS and 0.3% TritonX was added and incubated overnight at 4°C. The following antibodies were used: anti-Spry4 and anti-Oct4 (see Table 2 for dilutions). The slides were washed the following day twice with PBS containing 0.2% gelatin at room temperature and again twice with PBS only. A dilution of the second antibody in the blocking buffer was added and incubated at room temperature for 45min. To remove unbound antibodies, the slides were again washed 3 times with PBS containing 0.2% gelatin and 3 times with PBS only. To visualize the nuclei, the cells were then incubated with DAPI diluted at 1/10000 in PBS for 2min and finally washed twice with PBS. The cells were then fixed and mounted using Immumount solution (Shandon, Pittsburg, USA) and coversliped (Roth, Karlsruhe). Samples were examined with a fluorescent microscope (Leica).

4.2.4.2 Whole-mount immunostaining of preimplantation embryos

Embryos were flushed in PBS and transferred to a depression slide. They were then fixed in 1mL of 4% paraformaldehyde for 15min. Afterwards, the embryos were washed twice with 1mL PBS for 5min and then the fixation was blocked with 1mL of 15mM Glycin for 5min. The slides were once more washed with 1mL PBS for 5min. The embryos were then permeabilized with 1mL 0.5% Triton X-100 for 10min at room temperature and washed 2 times with PBS for 5min. The embryos were then incubated for 30min in 1mL of blocking solution (2% FCS/ 3% BSA in PBS) followed by 2 washing steps with 1mL of PBS for 5min. A dilution of the first antibody in blocking buffer was added and incubated overnight at 4°C. The following antibodies were used: anti-Spry4, anti-Cdx2 and anti-Nanog (see Table 2 for dilutions). The embryos were washed twice the following day with PBS containing 0.1% Triton X-100 and

2% serum for 20min at room temperature. A dilution of the second antibody in the blocking buffer was added and incubated at room temperature for 1h. To remove unbound antibodies, the slides were again washed 2 times with PBS containing 0.1% Triton X-100 and 2% serum for 20min at room temperature. To visualize the nuclei, the embryos were then incubated with DAPI diluted at 1/10000 in PBS for 2min and finally washed twice with PBS containing 0.1% Triton X-100 and 2% serum for 20min at room temperature. The blastocysts were then fixed and mounted using Immumount solution (Shandon) and coversliped (Roth). Samples were examined with a fluorescent microscope (Leica).

Abbreviations

ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
bp	base pair
cDNA	complementary Desoxyribonucleic acid
DAPI	4',6'- Diamidino-2-Phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamin-N,N-tetracetat
e.g.	Example given
EGTA	Ethylene glycol-bis(2-aminoethyl ether)-tetraacetate
FCS	Fetal calf serum
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	Hour
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.e.	*Id est* = that means
IMDM	Iscove's modified Dulbecco's medium
kb	Kilobase
kDA	Kilodalton
KO	Knockout
M	Molar
min	Minutes
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Secondes
TEMED	Tetramethylethylendiamine
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol
U	Unit
WT	Wild type

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Publications

Publication

Christna Chap, Gitta Blendinger and Ulrike Ziebold. Sprouty4 balances pluripotency and trophectoderm differentiation in embryonic stem cells. *Submitted*.

Talk and poster presentations

Christna Chap and Ulrike Ziebold. Defining the undifferentiated state of mouse embryonic stem cells. 9th MDC/FMP PhD retreat, Joachimsthal-Germany, Sept. 2007 (Talk).

Christna Chap, Maria Törnkvist and Ulrike Ziebold. An enhancer trap screen to identify regulators of differentiation in mouse embryonic stem cells. 8th MDC/FMP PhD retreat, Motzen-Germany, Sept. 2006 (Poster presentation).

Christna Chap, Maria Törnkvist and Ulrike Ziebold. An enhancer trap approach to screen embryonic stem cells to identify regulators of differentiation. International Symposium on Epithelial Organization and Organ Development, MDC Berlin-Germany, Mai 2006 (Poster presentation).

Statement

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständing und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Diese Arbeit wurde in gleichen oder ähnlicher Form nicht in anderen Prüfungsbehörde vorgelegt. Die Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 01. September 2005 habe ich gelesen und akzeptiert.